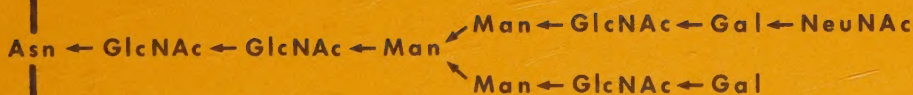
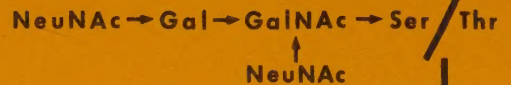


Structure and Properties of Some Glycoproteins Isolated from Human Urine

by

Birgitta Ekström



Lund 1980

STRUCTURE AND PROPERTIES OF SOME GLYCOPROTEINS ISOLATED FROM HUMAN URINE

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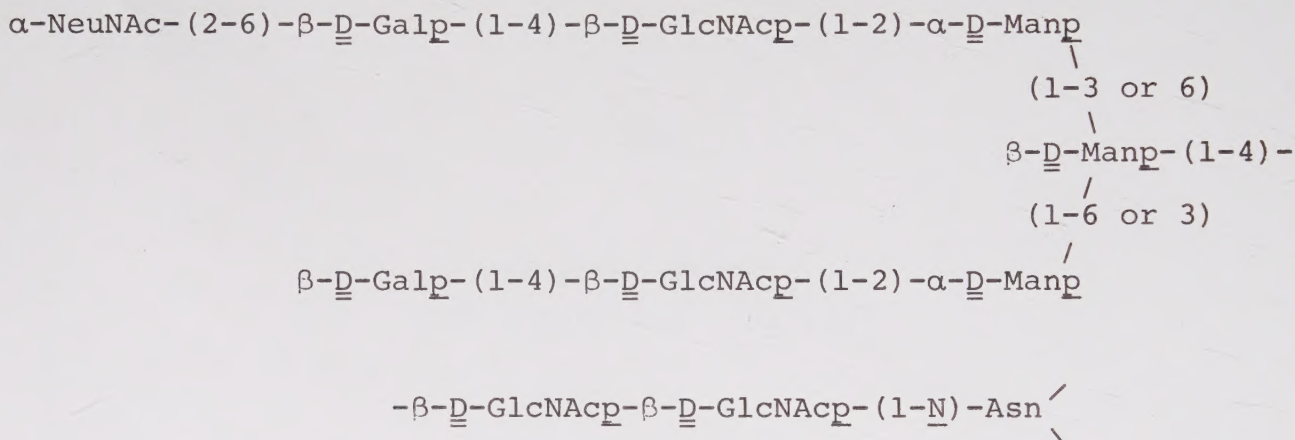
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SUMMARY

A glycoprotein (α_1 -microglobulin) with a molecular weight of about 26,000 has been isolated from the urine of patients with renal tubular proteinuria. This glycoprotein also occurs in small amounts in normal urine and plasma. The latter also contains high molecular weight proteins which react with rabbit anti-urine- α_1 -microglobulin serum.

A light brown chromophore is tightly bound to the urinary glycoprotein, which is homogeneous on ultracentrifugation, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate, and immunodiffusion. Charge heterogeneity is evident and apparently resides in the protein or chromophore. α_1 -Microglobulin contains 17% carbohydrate, and the structure of this part of the molecule was determined by trifluoroacetolysis, chromium trioxide oxidation, and Smith degradation. The starting material and degradation products were identified by sugar and methylation analysis. As a result, the following structure is proposed for the carbohydrate part of the molecule:-



It is calculated that three such chains are present on each glycoprotein molecule.

Three sialic acid-rich fractions (S_1 , S_2 , and S_3) with molecular weights of approximately 77,900, 37,000, and 5,300, respectively, have been isolated from the non-dialyzable material of normal human urine. Each fraction contains about 65% carbohydrate of which up to a half is sialic acid. The alkali-labile oligosaccharides of fraction S_3 have been characterized by sugar and methylation analysis, and were shown to have the same structure as the alkali-labile sugar chains of the M and N blood group antigens, namely α -NeuNAc-(2-3)- β -D-Galp-(1-3)-[α -NeuNAc-(2-6)-]-D-GalNAcp-(1-O)-Ser/Thr.

Some of these glycoproteins may originate from cell membranes, although the higher molecular weight material probably comes from mucin-producing cells of the kidney and urogenital tract.

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ISOLATED FROM HUMAN URINE

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Lund 1980

THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY
THE DEPARTMENT OF CLINICAL CHEMISTRY

UNIVERSITY OF TORONTO, TORONTO, CANADA



FELIX QUI POTUIT RERUM

COGNOSCERE CAUSAS

(*Vergilius*)

This dissertation is based on the following communications which will be referred to by the Roman numerals I-IV.

- I. A urinary and plasma α_1 -glycoprotein of low molecular weight: Isolation and some properties
B. Ekström, P.A. Peterson and I. Berggård
Biochem. Biophys. Res. Commun. 65 (1975) 1427-1433
- II. Human α_1 -microglobulin. Purification procedure, chemical and physicochemical properties
B. Ekström and I. Berggård
J. Biol. Chem. 252 (1977) 8048-8057
- III. Structural studies on the carbohydrate portion of human α_1 -microglobulin
B. Ekström, A. Lundblad and S. Svensson
Submitted for publication
- IV. Isolation and characteristics of human urinary sialoglycoproteins
I. Berggård, B. Ekström, E. Lisowska and A. Lundblad
Submitted for publication

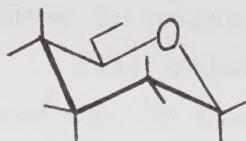
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Abbreviations used.

NeuNAc, N-acetylneuraminic acid; D-Gal, D-galactose; Galp, galactopyranose; D-GalNAc, 2-acetamido-2-deoxy-D-galactose; GalNAcp, 2-acetamido-2-deoxy-galactopyranoside; D-GalNAc-OL, 2-acetamido-2-deoxy-D-galactitol; D-GlcNAc, 2-acetamido-2-deoxy-D-glucose; GlcNAcp, 2-acetamido-2-deoxy-glucopyranose; D-GlcNAc-OL, 2-acetamido-2-deoxy-D-glucitol; D-Man, D-mannose; Manp, mannopyranose; Man-OL, mannitol; SDS, sodium dodecyl sulphate; GLC-MS, gas-liquid chromatography-mass spectrometry; CD, circular dichroism.

4C_1 conformation



1. INTRODUCTION

A large number of different glycoproteins are present in normal human urine (1-9). Many of them are derived from plasma, and at least one (the Tamm-Horsfall glycoprotein) from the renal tubular cells, but the origin of most of them is not known. In general they are considered to represent products of degradation of different tissue macromolecules. Only a limited number of the urinary glycoproteins have been characterized in detail.

Proteins and glycoproteins that are normally reabsorbed and catabolized by the renal tubular cells can be isolated in large amounts from urine of patients with tubular proteinuria. β_2 -Microglobulin (10) and the retinol-binding protein (11) have been isolated from such urine. Another glycoprotein, α_1 -microglobulin was also first isolated from urine of such patients and later shown to be a normal plasma protein (I). Detailed qualitative and quantitative analytical data on the normal urinary proteins and glycoproteins, and knowledge about the renal handling of these molecules form an important basis for the understanding of conditions with increased excretion. Also this information has proved to be useful in the diagnosis of certain renal disorders. The structural studies of excreted degradation products give indirect information about different tissue macromolecules.

The main part of the present study deals with the isolation and characterization of α_1 -microglobulin. In addition some sialic acid-rich glycoproteins, present in normal human urine and provisionally denoted S_1 , S_2 , and S_3 have been investigated.

α_1 -Microglobulin was first described in 1975 (I) as a low molecular weight (M.W. 26,000) glycoprotein with a light brown colour. The name was given in view of its size and electrophoretic mobility. Since then four other groups have published data on this glycoprotein (12-15) and different names have been suggested; protein HC (12) and α_1 -microglycoprotein (13). Åkerström and Berggård (16) have isolated a homologue of human α_1 -microglobulin from urine of guinea pigs.

In the early sixties Berggård (17) isolated a sialic acid-rich glycoprotein fraction by ultrafiltration and zone electrophoresis. The material was heterogeneous and therefore further attempts have been made to isolate more homogeneous fractions. From such material three sialoglycoprotein fractions (S_1 , S_2 , and S_3) have been isolated. Some physical, chemical and serological properties have been studied.

2. ISOLATION AND CHARACTERIZATION OF HUMAN α_1 -MICROGLOBULIN (I, II, III)

2.1. Isolation

The α_1 -microglobulin was purified from urine of patients with renal tubular or glomerulo-tubular proteinuria caused by chronic cadmium poisoning.

The isolation procedure included ultrafiltration, gel chromatography on Sephadex G-100, zone electrophoresis in sodium barbital buffer, and ion exchange chromatography on DEAE-Sephadex, carried out essentially as described (10,11).

The α_1 -microglobulin appeared pure when tested by immunoelectro-

phoresis, Ouchterlony immunodiffusion, and SDS-polyacrylamide gel electrophoresis. Some of the physico-chemical properties of α_1 -microglobulin are listed in Tables I and II, and are discussed below.

2.2. Size and electric charge

The molecular weight of about 26,000 has been estimated by ultracentrifugation and determination of the Stokes' radius and by gel chromatography in 6 M guanidine-HCl (II,13). SDS-polyacrylamide gel electrophoresis gives an apparent molecular weight of 31,000 (II,12-15). The overestimation of the molecular weight by this method may be due to the fact that α_1 -microglobulin contains carbohydrate and that it has an elongated shape or a high degree of hydration. The charge-heterogeneity of α_1 -microglobulin has been observed by others and the protein migrates in the α_1 -region as a broad zone on agarose gel electrophoresis and on zone electrophoresis. Desialylated α_1 -microglobulin showed a similar heterogeneity as the untreated protein on agarose gel electrophoresis. Hence the charge heterogeneity seems to reside in the polypeptide chain or in the material responsible for the brown colour, and not in the carbohydrate portion.

2.3. Other properties

The frictional ratio of α_1 -microglobulin (Table I) is relatively high, 1.45, suggesting a high degree of hydration and/or an elongated shape. Therefore the α_1 -microglobulin is eluted together with macromolecules of a higher molecular weight when purified on gel chromatography.

TABLE I Physical and chemical properties of human α_1 -microglobulin

Sedimentation coefficient, $s_{20,w}^0$	2.35 S
Stokes' molecular radius, r_s	2.85 nm
Apparent diffusion coefficient, $D_{20,w}$	$75 \mu\text{m}^2 \cdot \text{s}^{-1}$
Frictional ratio, f/f_0	1.45
Molecular weight	
by sedimentation and diffusion coefficients	26,100
by gel chromatography in 6 M guanidine-HCl	24,800
by SDS-polyacrylamide gel electrophoresis	31,000
Absorption coefficient at 280 nm, ϵ	$4.72 \cdot 10^4 \text{ M}^{-1} \text{ cm}^{-1}$

TABLE II Amino acid composition of human α_1 -microglobulin

Amino acid	Residues/molecule
Aspartic acid	14.3
Threonine	16.9
Serine	10.3
Glutamic acid	21.7
Proline	13.5
Glycine	14.0
Alanine	9.3
Half-cystine	3.4
Valine	10.9
Methionine	4.5
Isoleucine	12.4
Leucine	11.0
Tyrosine	8.3
Phenylalanine	6.2
Lysine	10.6
Histidine	3.6
Arginine	9.7
Tryptophan	2.7
Total	183

Purified α_1 -microglobulin has a tendency to form dimers in water solutions and is eluted as two peaks upon gel chromatography. The first peak corresponds to a molecular weight about twice the molecular weight of the main peak. The monomers are apparently not forming covalently-linked dimers, since only one band corresponding to monomeric α_1 -microglobulin can be detected when submitting the dimer form to SDS-polyacrylamide gel electrophoresis without reducing agents and on starch gel electrophoresis in 8 M urea at acid pH.

The absorption spectrum of α_1 -microglobulin shows a plateau starting at approximately 300 nm and decreases slowly throughout the visible region. This indicates the presence of a constituent other than amino acids or carbohydrate. The absence of any obvious maxima, except for that at 278 nm, suggests that this extra substance is not homogeneous. The CD-spectrum of α_1 -microglobulin is typical for a protein containing the β -pleated sheet conformation, but lacking α -helices.

2.4. Amino acid composition

The amino acid composition of α_1 -microglobulin is listed in Table II. It is similar to that reported by others (13,14,18). The protein contains 2.3 half-cystine residues per molecule when measured as carboxymethylcysteine after reduction and alkylation in 6 M guanidine hydrochloride followed by dialysis, and 3.4 residues when estimated as cysteic acid after performic acid oxidation. The lower value obtained after reduction and dialysis may indicate the presence of a free, dialyzable cysteine molecule involved in an S-S-bond with a half-cystine residue. On urea-starch gel electrophoresis there was no

observed decrease in the mobility of the α_1 -microglobulin after reduction. This also suggests the absence of a disulphide loop unless it is very small. Seon and Pressman (13) have proposed that two S-S-bonds are present, since they found 3.7 half-cystine residues after reduction, alkylation and dialysis in 7 M guanidine hydrochloride.

2.5. Carbohydrate composition

α_1 -Microglobulin contains about 17% by weight of carbohydrate. The relative molar proportions of the sugar constituents in human α_1 -microglobulin by sugar analysis (19,20) were: $\underline{\underline{D}}$ -Man, 3.0; $\underline{\underline{D}}$ -Gal, 2.2; $\underline{\underline{D}}$ -GlcNAc, 3.7; and NeuNAc, (1.1). The D-configuration is assumed. Methylation analysis (21) was carried out before and after desialylation of the glycoprotein. The results are presented in Table III.

TABLE III Methyl ethers obtained from hydrolysates of methylated α_1 -microglobulin and desialylated α_1 -microglobulin

Sugars	T-value ^a SE-30	Relative molar proportions	
		α_1 -Micro- globulin	α_1 -Micro- globulin ^b
2,3,4,6- $\underline{\underline{O}}$ -Me- $\underline{\underline{D}}$ -Gal	1.07	1.0 (1) ^c	2.1 (2)
2,3,4- $\underline{\underline{O}}$ -Me- $\underline{\underline{D}}$ -Gal	1.82	0.7 (1)	<0.05 (0)
3,4,6- $\underline{\underline{O}}$ -Me- $\underline{\underline{D}}$ -Man	1.45	1.8 (2)	2.2 (2)
2,4- $\underline{\underline{O}}$ -Me- $\underline{\underline{D}}$ -Man	2.40	0.8 (1)	0.8 (1)
3,6- $\underline{\underline{O}}$ -Me- $\underline{\underline{D}}$ -GlcN(Me)Ac	4.15	) + (4) ^d	+ (4) ^d
3,6- $\underline{\underline{O}}$ -Me- $\underline{\underline{D}}$ -GlcNAc	4.32		

^a Retention times of the corresponding alditol acetates relative to 1,5-di- $\underline{\underline{O}}$ -acetyl-2,3,4,6-tetra- $\underline{\underline{O}}$ -methyl- $\underline{\underline{D}}$ -glucitol.

^b Desialylated.

^c Figures in parentheses represent nearest integer.

^d The relative molar proportions were calculated from the sugar analysis (see text).

The removal of sialic acid residues was carried out with neuraminidase, Vibrio cholerae (II) or by mild acid hydrolysis (22). From and the two methylation analyses and the anomeric specificity of the neuraminidase, the sialic acid should be terminal and α -linked to one of the galactose residues in the 6-position.

The carbohydrate portion of α_1 -microglobulin has thus been shown to contain:

<u>D</u> -Galp-(1-	1 residue
α -NeuNAc-(2-6)- <u>D</u> -Galp-(1-	1 residue
-4)- <u>D</u> -GlcNAcp-(1-	4 residues
-2)- <u>D</u> -Manp-(1-	2 residues
-3)- <u>D</u> -Manp-(1-	1 residue
 6 	

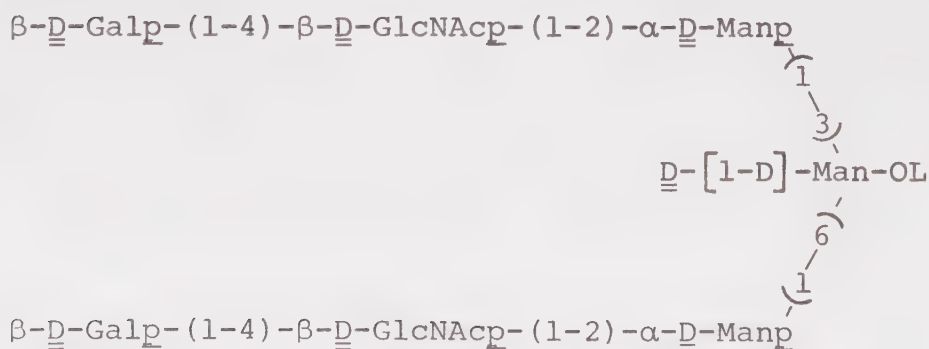
Trifluoroacetolysis (trifluoroacetic acid/trifluoroacetic anhydride, 1/1 (v/v), 100°C, 48 h) degrades glycoproteins by transamidation and releases the carbohydrate chains (23,24). The terminal 6-linked NeuNAc residues are removed quantitatively (25), and terminal reducing N-acetylhexosamines are partially degraded (23,24).

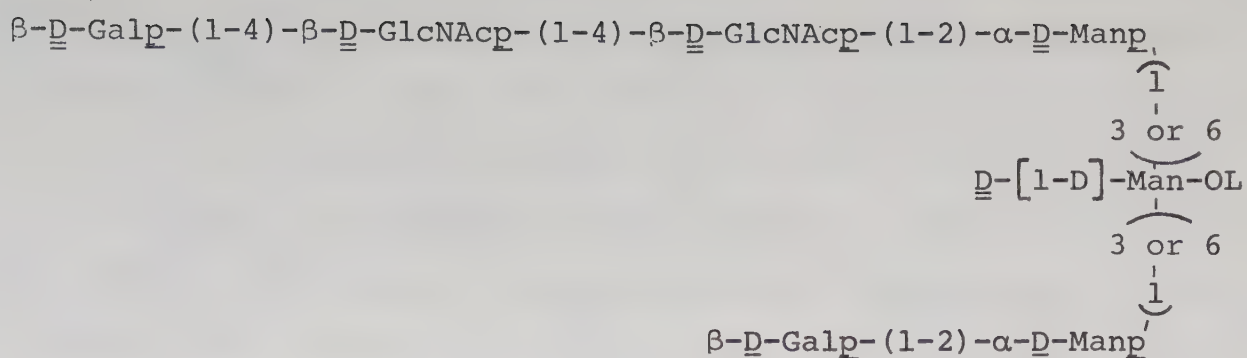
After trifluoroacetolysis the O-trifluoroacetyl groups were removed and N-trifluoroacetyl groups were replaced by N-acetyl groups. This material was purified by gel filtration and seemed homogeneous. Sugar analyses showed that no sialic acid residues were left, as expected. It also revealed that two GlcNAc residues had been degraded. Methylation analysis showed that the branched mannose residue was situated at the reducing end of the oligosaccharide obtained after the trifluoroaceto-

lysis degradation.

In order to determine the anomeric configuration of the sugars, the oligosaccharide was subjected to chromium trioxide oxidation (26). Free hydroxyl groups are attacked by the reagent but ester and amide bonds are stable, so the material was acetylated before oxidation. All the sugars present should have the 4C_1 conformation and hence the β -linkages should be attacked to yield 5-hexulosonates, whereas α -linkages should be essentially stable. Sugar analyses gave only traces of D-Gal and D-GlcNAc. D-Man and D-[1-D]-Man-OL were present in the ratio 2:1 in the oligosaccharide. Treatment with base cleaves the ester linkages in the 5-hexulosonates and after permethylation the presence of the trisaccharide α -D-Manp-(1-3)-[α -D-Manp-(1-6)-]D-[1-D]-Man-OL was established by comparison with an authentic sample using GLC-MS.

The oligosaccharide isolated after the trifluoroacetylotytic degradation may thus be assigned one of the structures $\underset{\sim}{1}$, $\underset{\sim}{2}$ or $\underset{\sim}{3}$.



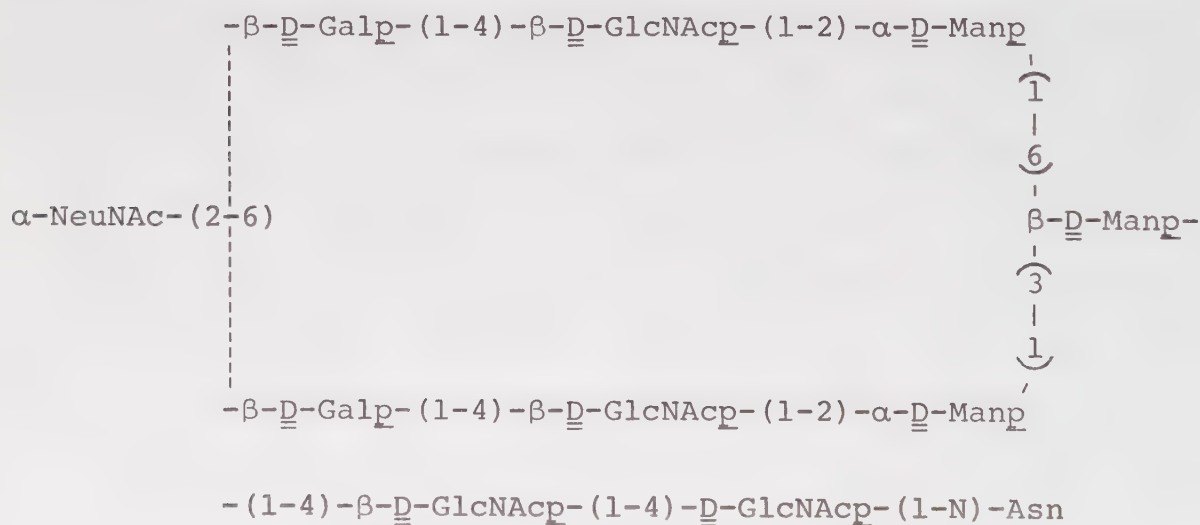
 ζ_2, ζ_3

Provided that the carbohydrate chains are homogeneous a Smith degradation (27) of α_1 -microglobulin should indicate the correct structure. After the oxidation and subsequent reduction (NaBH_4) and mild acid hydrolysis a dialyzable fraction was isolated, which was reduced (NaBD_4) and permethylated. On analysis by GLC-MS the only sugar derivative to be detected was permethylated β -D-GlcNAcp-(1-2)-[1-D]-glycerol which was identified by comparison with an authentic sample. Only structure 1 would be expected to give this fragment.

The non-dialyzable fraction was subjected to sugar and methylation analyses. D-Man and D-GlcNAc were present in the relative molar proportions 1:2, the D-Man residue being terminal and the D-GlcNAc residues substituted in the 1- and 4-positions. After subjecting the non-dialyzable fraction to trifluoro-acetolysis (trifluoroacetic acid/trifluoroacetic anhydride, 1/50 (v/v), 100°C, 48 h), the resulting oligosaccharides were fractionated on a Sephadex G-25 column (Fig. 1). Fractions A and B were subjected to sugar analysis. In fraction A D-Man and D-GlcNAc were present in the relative molar proportion 1:2 and in fraction B the relative molar proportion was 1:1.

Finally the anomeric configuration was investigated by measuring the optical rotation. Fractions A and B had an optical rotation of -53° and -59° , respectively, demonstrating that the two linkages between the three innermost sugars should both have the β configuration. The permethylated disaccharide alditol gave a mass spectrum similar to that of permethylated β -D-Galp-(1-4)-D-GlcNAc-OL. From these results it is evident that the structure of the components in fractions A and B is β -D-Manp-(1-4)- β -D-GlcNAcp-D-(1-4)-GlcNAc-OL and β -D-Manp-(1-4)-D-GlcNAc-OL, respectively.

From the molecular weight of α_1 -microglobulin and the known carbohydrate content it can be calculated that this glycoprotein has three identical carbohydrate chains per molecule having the structure



The proposed structure of the carbohydrate chains in α_1 -microglobulin has many features in common with the majority of the acidic asparagine-linked units found in several glycoproteins (27). These chains possess the trimannosyl central branching unit, α -D-Manp-(1-3)-[α -D-Manp-(1-6)-] β -D-Manp-(1-

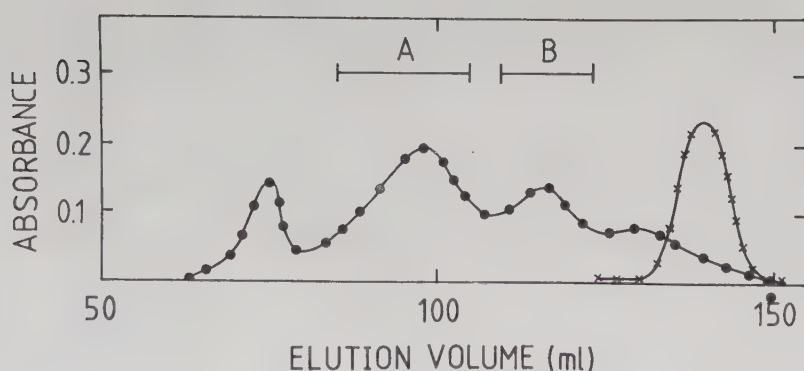


Fig. 1. Fractionation on a Sephadex G-25 column (1.5 x 80 cm) of the non-dialyzable (5 mg) product from Smith degradation - trifluoroacetolysis of α_1 -microglobulin. Eluted with water. Fractions (5.0 ml) were collected at 30 min intervals.

O—O Total hexose. X—X Sodium ion. Fractions were pooled as indicated in the figure. The void volume of the column was 74 ml.

and the di-N-acetylchitobiosyl-asparagine core. The peripheral chains, α -NeuNAc-(2-6)- β -D-Galp-(1-4)- β -D-GlcNAcp-(1-, are identical to those found in transferrin (28,29), immunoglobulins A, E, and G (30-32), and thyroxine-binding globulin (33) but differ from the chains of fetuin (23,34), which contain sialic acid 2-3-linked to the penultimate galactose. The carbohydrate units of α_1 -microglobulin differ from those found in the immunoglobulins by the lack of fucose and terminal N-acetylglucosamine (31), but are identical to the structure proposed for human plasminogen glycopeptide A (35).

2.6. Distribution of α_1 -microglobulin in biological fluids

Immunological analysis of human plasma revealed the presence of α_1 -microglobulin. When human plasma was chromatographed on a column of Sephadex G-200 (Fig. 2) four peaks of material reacting with rabbit anti-urinary α_1 -microglobulin serum were observed. The smallest peak corresponds in size to α_1 -micro-

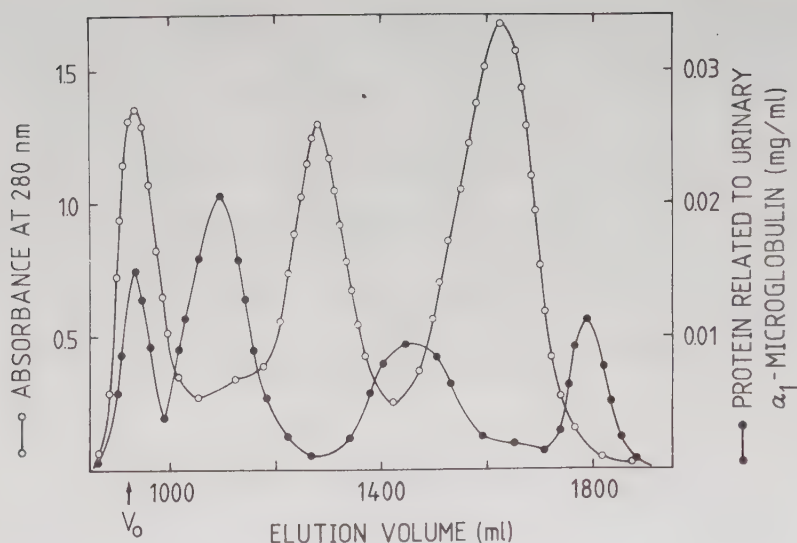


Fig. 2. Chromatography on Sephadex G-200 of 20 ml lipoprotein-depleted human serum. The column (150 x 4 cm) was equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.02% NaN_3 . Fractions of 8.7 ml were collected at a flow rate of 17.4 ml/h³. The distribution in the effluent of α_1 -microglobulin was determined by single radial immunodiffusion.¹

globulin from urine. Serum α_1 -microglobulin forms complexes with IgA and albumin (12). The IgA- α_1 -microglobulin complex is responsible for at least part of the second α_1 -microglobulin containing peak of the gel chromatogram (36). These different forms of α_1 -microglobulin make it difficult to obtain reliable estimates of the concentration of α_1 -microglobulin in normal human plasma.

Determination by single radial immunodiffusion gave a mean level of 54 mg/l, while the concentration was 98 mg/l when determined by Tejler and Grubb (12) and 32 mg/l according to Svensson and Ravnskov (14) using electroimmunoassay. The concentrations of α_1 -microglobulin in plasma from patients with renal failure was elevated, 298 mg/l, indicating the kidney as a main catabolic site. In blood from the umbilical cord the α_1 -microglobulin content, 26 mg/l, was lower than in serum from

adults. Cerebrospinal fluid contained only traces of α_1 -microglobulin.

The urinary excretion of α_1 -microglobulin in normal subjects has been estimated to be 9 mg/24 h using single radial immunodiffusion, and 10 mg/l (12) and 1.3 mg/24 h (14) using electroimmunoassay. Higher levels of urinary α_1 -microglobulin were found in patients with renal disorders.

α_1 -Microglobulin is a glycoprotein, binds to a brown-coloured substance and is charge-heterogeneous, but the significance of these properties remains to be determined. The biological role of α_1 -microglobulin is unknown.

3. PURIFICATION AND CHARACTERIZATION OF SOME HUMAN URINARY SIALOGLYCOPROTEINS (IV)

3.1. Purification

Non-dialyzable material from normal human urine was fractionated by zone electrophoresis in sodium acetate buffer pH 4.5 and then on gel chromatography on Sephadex G-200 (Fig. 3). Sialic acid and protein analyses revealed the presence of three main fractions designated S_1 , S_2 , and S_3 . Further purification was achieved by ion exchange chromatography on DEAE-cellulose in sodium acetate buffer pH 4.5 with a linear sodium chloride gradient. The yield of isolated sialoglycoproteins was 0.39 mg/l (S_1), 0.40 mg/l (S_2), and 1.9 mg/l (S_3). No corrections were made for loss of materials during the isolation and purification.

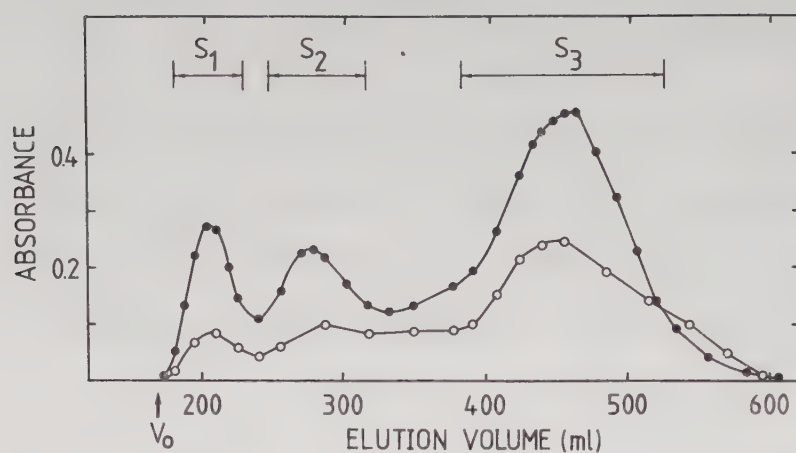


Fig. 3. Chromatography on Sephadex G-200 of sialic acid-rich material (194 mg) obtained by electrophoresis. The column (2.4 x 133 cm) was equilibrated with 0.5 M NaCl. Fractions of 7.5 ml were collected at 30 min intervals. V_0 indicates the void volume of the column. Eluates corresponding to fractions S_1 , S_2 , and S_3 were combined as shown on the figure. ●—● Sialic acid. ○—○ Protein.

TABLE IV Physical properties of sialoglycoproteins S_1 , S_2 , and S_3 .

Parameter	S_1	S_2	S_3
Sedimentation coefficient, $s_{20,w}^0$ (sec x 10^{13})	3.6	2.4	0.93
Stokes' radius (nm)	6.5	4.6	2.0
Diffusion coefficient, $D_{20,w}$ ($\text{cm}^2/\text{sec} \times 10^7$) ^a	3.25	4.6	10.6
Molecular weight			
by sedimentation equilibrium	77,900	37,000	5,300
by sedimentation-diffusion	74,900	35,500	5,900
Frictional ratio (f/f_0)	2.4	2.2	1.8
Absorption coefficient at 278 nm $10^3 \text{ M}^{-1} \text{ cm}^{-1}$	12.6	8.6	1.13

^a Apparent diffusion coefficient calculated from Stokes' radius.

3.2. Size

S_1 , S_2 , and S_3 behaved on ultracentrifugation as single and homogeneous components. A physico-chemical characterization of the three fractions has been made. Some data are given in Table IV. The molecular weights obtained by ultracentrifugation, sedimentation equilibrium and sedimentation-diffusion were similar. The frictional ratio of the three sialoglycoproteins is high (1.8-2.4), suggesting a high degree of hydration and/or an elongated shape.

3.3. Composition

The amino acid composition of the three sialoglycoproteins is listed in Table V. The compositions were different but some similarities were observed. Threonine and serine were found in high amounts. The content of tyrosine is low which also explains the low absorption coefficients at 278 nm. None of the sialoglycoproteins have any S-S-bonds or SH-groups in the polypeptide moiety. The S_3 fraction has a high content of aspartic acid and differs in this respect from other sialoglycoproteins isolated from normal urine by Goussault and Bourrillon (37) in which the contents of threonine and serine were higher.

The carbohydrate portion of the sialoglycoproteins (S_1 , S_2 , and S_3) represents 64 to 69% of the total weight (Table VI). The content of sialic acid is unusually high. The nature of the alkali-labile chains present in S_3 was studied. Two oligosaccharide fractions (B and C in Fig. 4) were isolated by alkaline borohydride degradation.

TABLE V. Amino acid composition of sialoglycoproteins S_1 , S_2 , and S_3 .

Constituent	S_1	S_2	S_3
	(residues per molecule)		
Lysine	3.93	3.17	0.11
Histidine	2.97	1.79	0.28
Arginine	1.49	1.46	0.30
Aspartic acid	8.70	7.88	3.04
Threonine	35.7	17.7	2.92
Serine	28.4	12.5	1.39
Glutamic acid	17.4	12.3	2.26
Proline	21.8	13.1	1.60
Glycine	11.2	5.09	1.04
Alanine	16.1	6.75	0.96
Half-cystine	-	trace	trace
Valine	9.08	4.51	1.49
Methionine	3.53	0.74	0.08
Isoleucine	4.73	2.25	0.21
Leucine	9.46	4.94	0.83
Tyrosine	0.89	0.67	0.31
Phenylalanine	0.83	0.61	0.56
Total	176	95	17.4

TABLE VI. Content of polypeptide and carbohydrate in sialoglycoproteins S_1 , S_2 , and S_3 .

Constituent	S_1	S_2	S_3
	(g per 100 g) ^a		
Polypeptide moiety ^b	23	27	35
Carbohydrate moiety	66	69	64
Total hexose	23.6	11.6	17.0
Hexosamine ^c	9.3	22.2	18.4
Sialic acid	32.7	35.2	28.4

^a All figures, except those for the polypeptide moieties of S_1 and S_2 , are average values from analyses of two preparations.

^b Calculated from quantitative amino acid analyses.

^c As N-acetyl derivative.

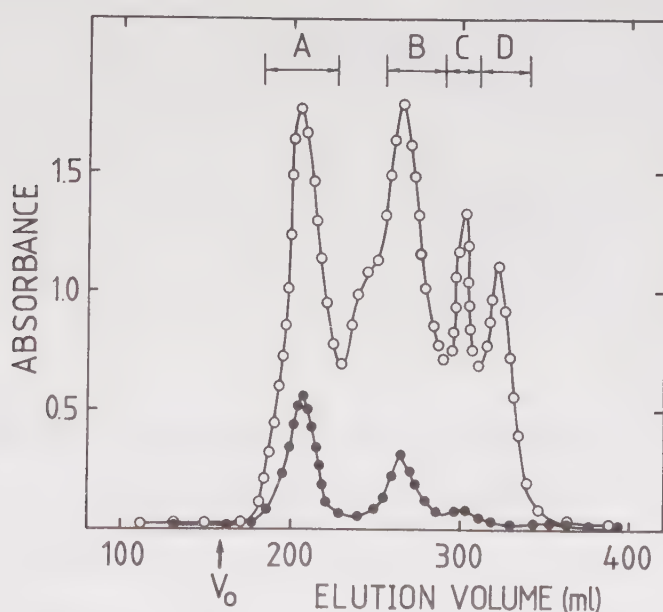
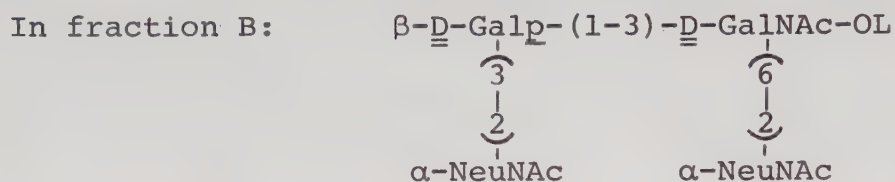


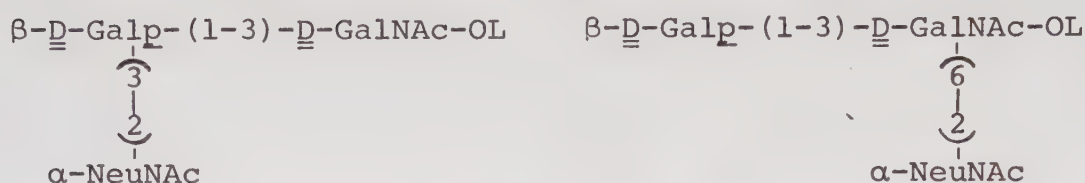
Fig. 4. Gel filtration on Sephadex G-25 of alkaline borohydride treated sialoglycoprotein fraction S_3 . The sample (31 mg) was placed on a column (2 x 130 cm), equilibrated with 0.1 M pyridine acetate buffer, pH 5.0. Fractions of 5.8 ml were collected at a flow rate of 10 ml/h. V_0 indicates the void volume of the column.

Eluates corresponding to fractions A, B, C, and D were combined as shown on the figure. ●—● Sialic acid. O—O Total hexose.

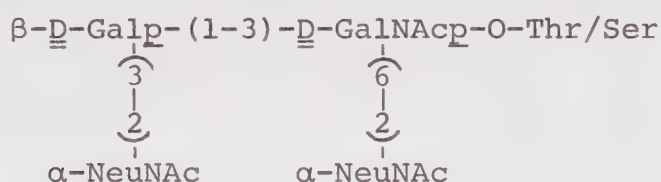
Sugar and methylation analyses of these fractions suggest the following structures for the main compound in each of these fractions:-



In fraction C, a mixture of the trisaccharides



The tetrasaccharide in fraction B is the same as the carbohydrate structure shown for alkali-labile chains of M and N blood group antigens (38-40):



3.4. Possible origin

The sialoglycoproteins studied here appear to belong to the mucin type of glycoproteins which can be highly fucosylated, the so called 'fucomucins', or highly sialylated ('sialomucins'). Between these two extremes all types of intermediate forms exist, and as a result of this a considerable heterogeneity is created. 'Fucomucins' have previously been studied in normal human urine (41,42) and the same type of heterogeneity was observed.

It should also be noted that these mucins can be a significant contaminant when attempts are made to isolate plasma type glycoproteins from normal urine in which their concentration is not particularly high. Some of the sialoglycoproteins may originate from cell membranes and similarities with M and N blood group glycoproteins would support such a hypothesis. However, the substances with higher molecular weight most likely originate from mucin producing cells in the kidney and the urogenital tract.

4. ACKNOWLEDGEMENTS

These investigations were initiated at the Department of Physiological Chemistry and completed at the Department of Clinical Chemistry, University of Lund, and I wish to thank Professor Bengt Borgström and Professor Per-Arne Öckerman for the facilities placed at my disposal. I wish to express my gratitude to my teachers, the late Professor Ingemar Berggård, and Drs. Arne Lundblad and Sigfrid Svensson, without whose knowledge and fundamental scientific interest these investigations would not have been undertaken. I also thank all co-workers and colleagues at the two departments for stimulating collaboration and assistance. I am indebted to Dr. Alan Chester for linguistic help. In addition I want to express my gratitude to Miss Kerstin Ohlson for expert secretarial help in preparing this thesis.

This work was financially supported by the Swedish Medical Research Council (13X-512, 03X-2, and 03X-4956), the Medical Faculty, University of Lund, Kungliga Fysiografiska Sällskapet, Lund, and the Swedish Society for Medical Research.

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A URINARY AND PLASMA α_1 -GLYCOPROTEIN OF LOW MOLECULAR WEIGHT:
ISOLATION AND SOME PROPERTIES

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SUMMARY: A low molecular weight α_1 -glycoprotein (α_1 -microglobulin) was purified from urine of patients with tubular proteinuria and was found to occur in low concentrations in normal human plasma. Plasma also contains high molecular weight proteins which react with anti- α_1 -glycoprotein sera. The purified protein appeared homogeneous when subjected to ultracentrifugation and immunodiffusion analyses. On agarose gel electrophoresis, it gave an electrophoretically heterogeneous broad band in the slow α_1 -region. The α_1 -glycoprotein was found to have a molecular weight of about 25,000 and a carbohydrate content of 23 %. It appears to consist of a single polypeptide chain. An unidentified colored material is strongly bound to the protein.

INTRODUCTION: Plasma proteins with molecular weights below 40,000 are present only in low concentrations in blood from healthy individuals. Such proteins seem to pass the glomerular barrier in the kidney relatively easily and are then apparently resorbed and catabolized in the renal tubules. Only small amounts of plasma proteins are normally excreted in the final urine (1). Patients with tubular proteinuria due to renal tubular resorption defects excrete considerable amounts of low molecular weight plasma proteins, such as β_2 -microglobulin (2) and the retinol-binding protein (3).

The present paper describes a low molecular weight α_1 -glycoprotein isolated from urine of patients with chronic cadmium poisoning, a condition known to be associated with tubular proteinuria (1). This apparently novel α_1 -glycoprotein was found in small quantities in normal human plasma and urine, but in considerably larger amounts in the urine from patients with tubular proteinuria. It is electrophoretically heterogeneous and contains a tightly linked colored material. Human plasma also contains three high molecular weight proteins with an immunological reactivity similar to that of the α_1 -glycoprotein.

MATERIALS AND METHODS: Twenty-four-hour urine specimens were obtained from healthy individuals and from patients with tubular or mixed glomerular-tubular proteinuria caused by chronic cadmium poisoning. Urine collections and sample processing were carried out as described elsewhere (2).

Ultrafiltration, gel chromatography on Sephadex G-100, zone electrophoresis in sodium barbital buffer, and ion exchange chromatography on DEAE-Sephadex were carried out essentially as described (2, 3). Antisera were prepared in rabbits. The immunization procedure has been described (2). Agarose gel electrophoresis in 0.075 M sodium barbital buffer, pH 8.6, was performed according to Johansson (4). Half-cysteine was estimated as cysteic acid after performic acid oxidation (5). Measurement of molecular weight by gel chromatography on Sepharose-6B in 6 M guanidine hydrochloride was carried out according to Fish et al. (6). The column was calibrated with reference proteins (7). Free sulfhydryl groups were estimated by spectrophotometric titration (8) with 5,5' dithiobis (2-nitrobenzoic acid) or by alkylation with ^{14}C -iodoacetamide (9). Digestion with neuraminidase was performed in 0.1 M acetate buffer, pH 5.0, using sialic acid:neuraminidase ratios of 1:2 (w/w). The digestion was stopped by lyophilization. Other analytical methods have been described (2, 3).

RESULTS AND DISCUSSION: The initial preparation of urinary α_1 -glycoprotein was isolated essentially as described below. Antisera against this preparation were raised in rabbits. These antisera were used in the single radial immunodiffusion technique to trace and quantitate the protein during the subsequent purification work.

Concentrated urinary macromolecules were separated on columns of Sephadex G-100. Several peaks of protein were observed. The α_1 -glycoprotein was present in a high protein peak (Fig. 1) which also contains

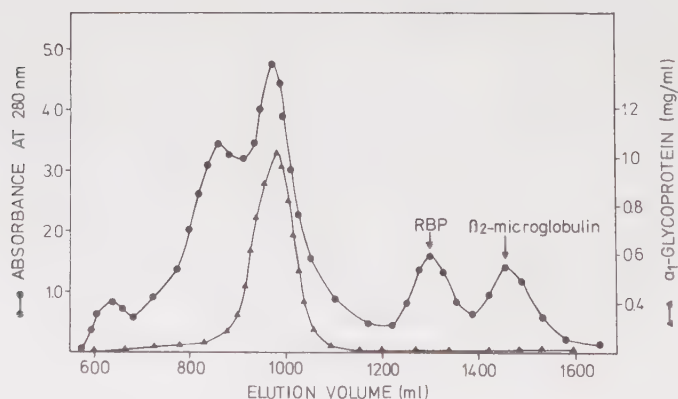


Fig. 1. Chromatography on Sephadex G-100 of concentrated urinary macromolecules from seven patients with chronic cadmium poisoning. The column (104 x 5 cm) was equilibrated with 0.02 M Tris-HCl buffer, pH 7.8, containing 1.0 M NaCl. The applied sample (19 ml) contained 1210 mg of total protein and 194 mg of α_1 -glycoprotein. Fractions of 8 ml were collected at a flow rate of 24 ml per hour. The distribution of protein in the effluent was determined by reading the absorbancy at 280 nm (●—●), and α_1 -glycoprotein was measured by the single radial immunodiffusion technique (▲—▲). The peaks of retinol-binding protein (RBP) and of β_2 -microglobulin are indicated.

free immunoglobulin light chains (10). This peak from one or from several Sephadex G-100 separations was pooled and concentrated by ultrafiltration. In one of the preparations, concentrated urine which contained 5200 mg of protein and 850 mg of α_1 -glycoprotein gave a fraction containing 1290 mg of total protein and 530 mg of α_1 -glycoprotein. This material was subjected to zone electrophoresis in 0.1 M barbital buffer, pH 8.6. An anodal fraction with α_1 -glycoprotein and a cathodal fraction with free immunoglobulin light chains were obtained. The α_1 -glycoprotein was chromatographed on a Sephadex G-100 column. It emerged as a symmetrical peak after small amounts of impurities of larger molecular size. The α_1 -glycoprotein was finally purified by chromatography on DEAE-Sephadex. The column, equilibrated with 0.05 M Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl, was eluted with a linear gradient of sodium chloride, from 0.1 to 0.3 M. The α_1 -glycoprotein was eluted as a somewhat asymmetrical peak suggesting charge heterogeneity. Fractions corresponding to the main part of the peak were dialyzed against distilled water and lyophilized. In three preparations, the yields of purified α_1 -glycoprotein were 9-10 % of the amounts present in the concentrated urinary proteins. The lyophilized protein had a brown color, and the α_1 -glycoprotein could, in fact, be traced visually by its brown color during the entire isolation procedure.

The purity of the α_1 -glycoprotein was assessed by immunoelectrophoresis and by Ouchterlony immunodiffusion analyses. Only a single precipitation line was observed when the α_1 -glycoprotein was tested against a polyvalent anti-urinary protein serum. This antiserum precipitated a large number of proteins in the starting material.

The occurrence of α_1 -glycoprotein in normal plasma and in concentrated normal urine was established by Ouchterlony immunodiffusion analyses using anti- α_1 -glycoprotein serum. There was a reaction of identity between the plasma and urine components and purified α_1 -glycoprotein. To examine plasma component(s), lipoprotein-depleted plasma was chromatographed on a column of Sephadex G-200 (Fig. 2). It was necessary to concentrate the fractions from this experiment in order to trace the protein by immunochemical gel diffusion techniques. Four peaks were detected with the single radial immunodiffusion technique. The last-eluted peak emerged in the position of urinary α_1 -glycoprotein. Three peaks were eluted earlier, obviously owing to larger components. Urinary α_1 -glycoprotein was used as the standard; therefore the single radial immunodiffusion curve does not show the true relative quantities of the four plasma components. The four components could be demonstrated also when α_1 -glycoprotein was traced by radioimmunoassay. Fresh plasma and plasma that had been stored at -20° for several

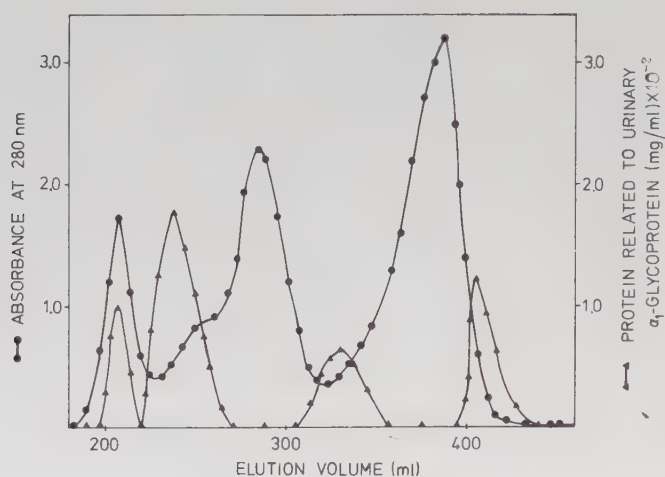


Fig. 2. Chromatography on Sephadex G-200 of lipoprotein-depleted plasma (5 ml). The column (140 x 2.4 cm) was equilibrated with 0.02 M Tris-HCl buffer, pH 7.8, containing 0.15 M NaCl. The flow rate was 9 ml per hour. Concentrations of total protein (●-●) and of protein reacting with anti-urinary α_1 -glycoprotein serum (▲-▲) were measured as described in the legend of Fig. 1. Before immunochemical analyses, fractions were concentrated 25-50 times by means of Minicon-B15 ultrafilters (Amicon).

months gave similar chromatographic patterns. In immunoelectrophoretic experiments, the smallest plasma component and the protein in normal urine appeared to have the same mobility as the purified α_1 -glycoprotein. The larger size plasma components had a lower electrophoretic mobility.

The excretion of α_1 -glycoprotein in normal urine and in urine from patients with chronic cadmium poisoning was determined by the single radial immunodiffusion technique. Urine from five healthy individuals contained 0.75 to 2.9 mg of the protein per 24-hour volume, whereas urine from seven patients with tubular proteinuria contained 110 to 250 mg.

The α_1 -glycoprotein appeared homogeneous on ultracentrifugation, as sedimentation velocity analyses revealed a single component which remained symmetrical throughout the experiments. On agarose gel electrophoresis at pH 8.6, it gave an electrophoretically heterogeneous, broad band in the slow α_1 -region (Fig. 3).

Table 1 gives some physical and chemical properties of α_1 -glycoprotein. The sedimentation coefficient was independent of protein concentrations ranging from 1.25 to 10 mg per ml. The molecular weight measured by sedimentation equilibrium ultracentrifugation, and the molecular weight found by gel chromatography in 6 M guanidine-HCl after reduction and alkylation,

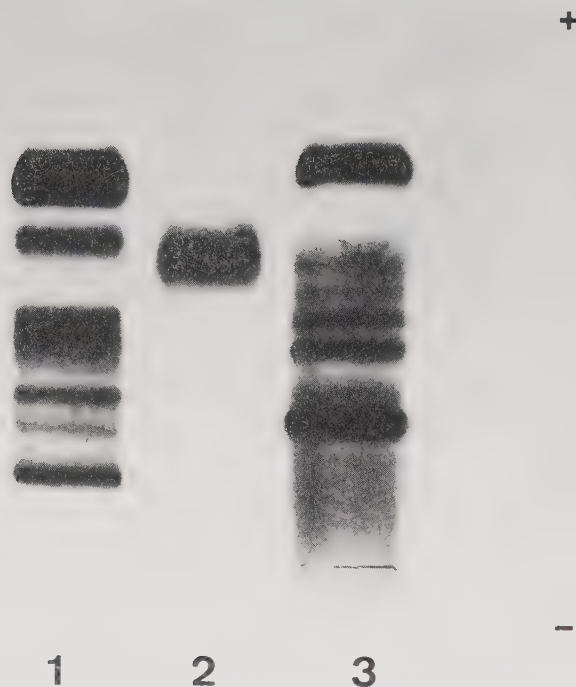


Fig. 3. Agarose gel electrophoresis in 0.075 M barbital buffer, pH 8.6, of normal plasma (1), purified α_1 -glycoprotein (2), and urinary proteins from a patient with chronic cadmium poisoning (3).

seem to be identical within experimental error. A relatively large part of the molecule is composed of carbohydrate (23 %). To determine whether the electrophoretic heterogeneity of the protein depends on a varying number of sialic acid residues, digestion with neuraminidase was performed. On agarose gel electrophoresis, the neuraminidase-treated α_1 -glycoprotein moved more slowly, but showed an electrophoretic heterogeneity similar to the untreated protein. This suggests that the heterogeneity resides in the polypeptide chain or in the chromophore material. Amino acid analyses and determinations of sulfhydryl groups indicated that each molecule of α_1 -glycoprotein contains three cysteine residues and no disulfide bonds. It is most probable that the α_1 -glycoprotein is made up of a single polypeptide chain, as only one component with the molecular weight of the intact protein was observed on gel chromatography in 6 M guanidine hydrochloride. The chromophore material seems to be quite strongly bound to the protein, as it was not removed by the various isolation procedures or by gel chromatography in 6 M guanidine hydrochloride.

Table 1. Some physical and chemical properties of human urinary α_1 -glycoprotein

Sedimentation coefficient, $s_{20,w}^0$	2.3 S
Molecular weight	
By sedimentation equilibrium ultracentrifugation	24,400
By gel chromatography in 6 M guanidine hydrochloride	25,000
Polypeptide moiety ^a	78 %
Carbohydrate moiety ^b	23 %
Neutral sugar	6.3 %
Hexosamine ^c	11.6 %
Sialic acid	5.2 %
Fucose	0.13 %

^a Calculated from quantitative amino acid analyses.

^b All carbohydrate figures are average of values from at least two separate determinations.

^c As N-acetyl derivative.

One question posed by the present observations is whether the α_1 -glycoprotein isolated from urine is identical with the corresponding protein found in normal plasma. The urinary α_1 -glycoprotein and the low molecular weight plasma component are probably identical, as they have the same or a very similar antigenic structure, molecular size, and electrophoretic mobility at pH 8.6. Normal plasma also contains three components of higher molecular weight which react with anti- α_1 -glycoprotein serum. Accordingly, the α_1 -glycoprotein might be a subunit of these larger, probably unknown, plasma proteins.

In accordance with previously used nomenclature (2) we propose that, for the present, the above-described protein is called α_1 -microglobulin.

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Human α_1 -Microglobulin

PURIFICATION PROCEDURE, CHEMICAL AND PHYSICOCHEMICAL PROPERTIES*

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A human α_1 -glycoprotein of low molecular weight has been isolated from urine of patients with tubular proteinuria and shown to be present in serum and urine of healthy individuals. The isolation procedure includes ultrafiltration, gel chromatography, zone electrophoresis, and ion exchange chromatography. The yield of purified protein was 10 to 15% of the amount in concentrates of urinary proteins.

The isolated protein appeared pure on immunodiffusion analyses and homogeneous when subjected to ultracentrifugation and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Charge heterogeneity was observed by other electrophoretic techniques. At pH 8.6 the main part of the protein migrated in the slow α_1 -globulin region. α_1 -Microglobulin has a brown color due to a tightly linked and unidentified chromophore material. The protein apparently consists of a single polypeptide chain that has some tendency to form noncovalently linked dimers. The molecular weight as determined by sedimentation equilibrium ultracentrifugation was 26,700, by sedimentation-diffusion data 26,100, and by gel chromatography in 6 M guanidine hydrochloride 24,800. Carbohydrate analyses revealed close to equimolar amounts of glucosamine, mannose, galactose, and sialic acid with a total carbohydrate content of 20%. The protein was also heterogeneous in charge after removal of sialic acid residues by digestion with neuraminidase. This suggests that the heterogeneity resides in the polypeptide chain or in the chromophore material. A relatively high frictional ratio (1.45) indicated that the molecule has an elongated shape, or a high solvation, or both. Circular dichroism spectra suggested the presence of β -pleated sheet conformation and the absence of α helical structure.

Immunochemical determinations of α_1 -microglobulin gave the following mean values: normal serum, 54 mg/liter, serum of umbilical cord blood, 26 mg/liter, and normal urine, 9 mg/24-h urine volume. Both serum and urine from patients with renal diseases had greatly increased concentrations of α_1 -microglobulin. This finding and the small

size of the protein indicates that the kidney is its main catabolic site.

It is now well established that the smallest plasma proteins are eliminated mainly in the kidneys, apparently by glomerular filtration followed by tubular reabsorption and catabolism (1). Therefore, urine from patients with renal tubular reabsorption defects has been shown to be a particularly good source for isolation of small plasma proteins. We have previously purified β_2 -microglobulin and the retinol-binding protein from such urine (2, 3). Both are present in quite low concentrations in the blood.

During these studies we observed an apparently novel, brown-colored protein which is described in the present paper. After isolation of this small protein from urine, we found that it is also present in several forms in plasma. In view of its size and electrophoretic mobility, and in accordance with previously used nomenclature (2), we call it α_1 -microglobulin. The α_1 -microglobulin is a glycoprotein with a charge-heterogeneity that apparently resides in the peptide or chromophore parts of the molecule.

A preliminary report of parts of this work has been published (4). Subsequently, some properties of α_1 -microglobulin have been studied also by two other groups (5-8). One of the groups has called it protein HC: human complex-forming glycoprotein, heterogeneous in charge (5).

EXPERIMENTAL PROCEDURES

Materials

Urines—Twenty-four-hour urine specimens were obtained from healthy individuals and from patients with tubular or mixed glomerular-tubular proteinuria due to chronic cadmium poisoning. Urine collection and sample processing were carried out as described previously (2).

Sera—Portions of serum from healthy blood donors were obtained from the Blood Center, University Hospital, Lund. Sera from patients with renal failure were given by Dr. Lars Wibell, Medical Clinic, University Hospital, Uppsala. Dr. Laila Ekelund, Department of Obstetrics and Gynecology, Malmö General Hospital, Malmö, furnished us with samples of sera from umbilical cord blood.

Cerebrospinal Fluids—The samples were the same as those used earlier (2); they had been stored at -20° .

Antisera—Antisera were raised in rabbits against highly purified α_1 -microglobulin, orosomucoid, transferrin, and albumin, and also against partially purified α_1 -microglobulin obtained by gel chromatography, zone electrophoresis, and ion exchange chromatography. Purified orosomucoid and transferrin were obtained from Behringwerke A.G. (Marburg, Germany) and albumin was a gift from AB

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Kabi (Stockholm, Sweden). The immunization procedure has been described (2).

Antisera raised against purified α_1 -microglobulin produced a single precipitation arc when tested on immunoelectrophoresis against varying amounts of concentrated urinary proteins. Similar tests with antisera against partially purified α_1 -microglobulin gave one strong arc and two or three fainter arcs.

Antisera against pooled concentrated urinary proteins from patients with tubular proteinuria were the same as those used earlier (2).

The following antisera were obtained from Behringwerke A.G. (Marburg, Germany): anti-inter- α -trypsin-inhibitor, anti- α_1 -anti-trypsin, anti- α_1 -B-glycoprotein, anti- α_1 -T-glycoprotein, and anti- α_1 -antichymotrypsin.

Other Materials—Sephadex G-100, Sephadex G-200, Sepharose CL-6B, and DEAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and prepared according to the instructions supplied. Pevikon C-870 was purchased from Kema Nord AB (Stockholm, Sweden) and partially hydrolyzed starch from Connaught Laboratories (Toronto, Canada). Agarose (Indubiose A-45) was a product of L'Industrie Biologique Française S.A. (Gennevilliers, France). Iodo[14 C]acetamide was obtained from New England Nuclear Corp. (Boston, Mass.) and 125 I from Amersham (Buckinghamshire, England). All other chemicals used were reagent grade or of highest available quality.

Methods

Concentration of Proteins—Urine and protein solutions were concentrated by ultrafiltration with 23/32-inch dialysis tubing (Union Carbide Corp., Chicago, Ill.) (9). The concentrates were processed immediately or stored at -20° .

Electrophoretic Methods—Preparative zone electrophoresis in blocks of Pevikon C-870, polyacrylamide gel electrophoresis in slabs, and starch gel electrophoresis in 8 M urea and formate buffer were carried out with previously reported techniques (3). For analysis by starch gel electrophoresis, some samples of protein, dissolved in 0.2 M Tris/HCl buffer, pH 8.0, containing 8 M urea, were reduced with 0.1 M 2-mercaptoethanol for 30 min and thereafter alkylated with 0.12 M iodoacetamide for 30 min.

Disc polyacrylamide gel electrophoresis in sodium dodecyl sulfate was done according to the method of Neville (10). Both unreduced α_1 -microglobulin and α_1 -microglobulin reduced as described (11) were examined. β_2 -Microglobulin, myoglobin, lactate dehydrogenase, ovalbumin, albumin, and light and heavy chains of IgG were used as reference proteins for molecular weight determinations.

Agarose gel electrophoresis was performed in 0.075 M sodium barbital buffer, pH 8.6, as described by Johansson (12).

Immunochemical Methods—Immunodiffusion in gel according to Ouchterlony, immunoelectrophoresis, and single radial immunodiffusion were carried out as reported (2).

Ultracentrifugation—Sedimentation velocity and equilibrium experiments were carried out at 20° in a Beckman model E analytical ultracentrifuge equipped with an RTIC temperature control unit and an electronic speed control system (Beckman Instruments, Palo Alto, Calif.). All experiments were conducted in 0.02 M Tris/HCl buffer, pH 8.0, containing 0.15 M NaCl. Standard 12-mm double-sector cells with sapphire windows were used throughout and the speed for the sedimentation velocity measurements was 48,000 rpm. The sedimenting boundary was photographed every 16 min with use of the phase plate schlieren optics. Calculations were carried out according to the method of Schachman (13).

Sedimentation equilibrium experiments were performed with the meniscus depletion technique of Yphantis (14). Recordings were made with Rayleigh interference optics. The appropriate speed, 30,000 rpm, was chosen as suggested by Yphantis (14). To determine whether equilibrium had been established, the fringe displacements at given X coordinates were measured on photographs taken at different time intervals. The experiments were interrupted when no significant fringe shift was observed over a period of several hours.

The partial specific volume for the calculations was derived from the amino acid and carbohydrate composition reported below (15, 16).

Determination of Molecular Weight by Gel Chromatography—Untreated and reduced and alkylated α_1 -microglobulin was subjected to gel chromatography on a Sepharose CL-6B column (120×1.5 cm), equilibrated with 0.05 M sodium acetate buffer, pH 4.8, containing 6 M guanidine hydrochloride (11). Two of the three preparations examined were labeled with 125 I before analysis. In these experiments radioactivity was determined in a γ counter. The column was

calibrated with the following reduced and alkylated reference proteins: heavy and light chains of IgG, albumin, ovalbumin, myoglobin, and β_2 -microglobulin. α_1 -Microglobulin and the reference proteins were reduced for 2 h in 0.2 M Tris/HCl buffer, pH 8.0, containing 6 M guanidine hydrochloride and 0.02 M dithioerythritol. The samples were alkylated for 30 min by addition of iodoacetamide to a molarity of 0.05.

The molecular weights of α_1 -microglobulin and an oligomeric form of this protein were compared by gel chromatography following the method of Andrews (17). The analyses were performed on a Sephadex G-200 column (107×0.9 cm) which was equilibrated with 0.02 M Tris/HCl buffer, pH 8.0, containing 0.15 M NaCl. IgG, albumin, chymotrypsinogen, and β_2 -microglobulin were used as reference proteins.

Estimation of Stokes Radius—Stokes molecular radius (r_s) was determined by gel chromatography according to the method of Laurent and Killander (18). The analyses were made at room temperature on a Sephadex G-200 column (107×0.9 cm) as described by Karlsson *et al.* (19). The column was equilibrated with 0.02 M Tris/HCl buffer, pH 8.0, containing 0.15 M NaCl.

Calculations of Apparent Diffusion Coefficient, of Molecular Weight by Svedberg Equation, and of Frictional Ratio—The apparent diffusion coefficient ($D_{20, w}$) was computed from Stokes radius by use of the Stokes-Einstein equation (20). Svedberg's equation was used to obtain the molecular weight from the diffusion coefficient and the sedimentation coefficient. The frictional ratio (f/f_0) was calculated from the sedimentation coefficient and the molecular weight determined by sedimentation equilibrium ultracentrifugation. The formulas used have been reported by Svedberg and Pedersen (21).

Circular Dichroism—CD spectra were measured with a Jasco model J-41A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) essentially as described by Björk *et al.* (22). α_1 -Microglobulin was dissolved at a concentration of 0.1 mg/ml in 0.15 M NaCl for spectra below 245 nm, and at a concentration of 0.8 mg/ml in 0.01 M phosphate buffer, pH 7.4, containing 0.15 M NaCl for spectra between 245 and 350 nm. The results are presented as reduced mean residue ellipticity against wavelength. The parameter $[\theta']$ was computed as described by Cathou *et al.* (23). The mean residue weight was calculated from the amino acid composition and was found to be 113.

Amino Acid Analysis—Quantitative amino acid analyses were carried out essentially as described by Spackman *et al.* (24). The protein samples (about 0.5 mg) were hydrolyzed in 6 M HCl at 110° for 24 and 72 h (25). Chromatography was carried out on a Durrum model D-500 amino acid analyzer according to the instructions supplied by Durrum, Palo Alto, Calif. Half-cystine was estimated as cysteic acid after performic acid oxidation (26), and as carboxymethylcysteine after reduction and alkylation in 6 M guanidine hydrochloride, as described above. Tryptophan was estimated spectrophotometrically by the procedure of Spande and Witkop (27).

Analysis of Free Sulfhydryl Groups—Free sulfhydryl groups were estimated by spectrophotometric titration with 5,5'-dithiobis(2-nitrobenzoic acid) (28) in 0.1 M sodium phosphate buffer, pH 7.4, containing 10 mM EDTA-disodium salt, and by alkylation with iodo[14 C]acetamide (29) in 0.2 M Tris/HCl buffer, pH 8.0, containing 6 M guanidine hydrochloride.

Determination of Carbohydrate—Hexosamines and neutral sugars were determined as alditol acetates by gas-liquid chromatography (30) and mass spectrometry (31) after hydrolysis in 4 M trifluoroacetic acid at 100° for 4 h (32). The α_1 -microglobulin was hydrolyzed with D-xylose as an internal standard. Hexosamines were also measured in the amino acid analyzer after hydrolyses for 24 h (see above). The recovery of hexosamines under the hydrolysis conditions used has been estimated at 44%.

Neutral sugars were also analyzed by an orcinol method (33). This method was used as delineated earlier (9). Sialic acid was estimated by the Bial reaction as described by Werner and Odén (34) and by the thiobarbituric acid assay (35). In all three colorimetric methods the absorption curve for the test solution was of the same shape as the absorption curve for the standard.

Digestion with Neuraminidase—Digestion with neuraminidase (Sigma) was performed in 0.1 M sodium acetate buffer, pH 5.0, using sialic acid:neuraminidase ratios of 1:2 (w/w). The digestion of 3 to 4 mg of α_1 -microglobulin in 3 ml of buffer was carried out at 37° for 24 h. The sample was dialyzed against 750 ml of the buffer during the incubation and against distilled water at 4° prior to lyophilizing.

Other Methods—Protein concentrations in unpurified materials

and in fractions obtained from the first purification step were measured by the modified Folin method of Lowry *et al.* (36) with human IgG as the standard. In more purified fractions protein was determined by reading the absorbance at 280 nm. The light absorption of α_1 -microglobulin in the ultraviolet and visible ranges was measured in 0.1 M sodium phosphate buffer, pH 7.0, on a Zeiss model PMQ II spectrophotometer. The molar extinction coefficient at 280 nm was obtained after corrections for content of ash and moisture. Moisture was measured by drying to constant weight at 100° and 0.05 mm Hg. Nitrogen analyses were carried out by the method of Dumas (37). Labeling with ^{125}I was accomplished by the chloramine-T procedure of Greenwood *et al.* (38).

RESULTS

Purification of α_1 -Microglobulin

α_1 -Microglobulin was isolated separately from several portions of concentrated macromolecules derived from both individual and pooled urine of patients with chronic cadmium poisoning. The preparations gave similar results and yields.

The first purification gave partially purified α_1 -microglobulin and was carried out by a procedure which resembled that described below. An antiserum was raised against this preparation and was used in the course of the subsequent purifications to trace α_1 -microglobulin and also two or three of the main contaminating proteins.

The purification procedure is described below and a typical series of experiments is summarized in Table I. The yields of purified α_1 -microglobulin were 10 to 15% of the amounts present in the concentrated urinary proteins. All operations were carried out at +4°.

First Gel Chromatography on Sephadex G-100—The concentrated urinary macromolecules were subjected to gel chromatography on a Sephadex G-100 column (113 × 8 or 104 × 5 cm) equilibrated with 0.02 M Tris/HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.02% NaN₃. Several peaks of protein were observed (Fig. 1). The eluates were assayed for protein by measurements of the absorbance at 280 nm and for α_1 -microglobulin and albumin by the single radial immunodiffusion technique. The main part of α_1 -microglobulin emerged somewhat later than albumin in a distinct peak, although some α_1 -microglobulin was always eluted earlier. A small peak of protein that reacts with anti- α_1 -microglobulin serum was seen at the void volume of the column in some of the separations. The large peak with α_1 -microglobulin also contains substantial amounts of free immunoglobulin light chains (39). Fractions containing α_1 -microglobulin were pooled as indicated in Fig. 1 and concentrated by ultrafiltration.

TABLE I
Purification of human α_1 -microglobulin

	Total protein	α_1 -Microglobulin ^a	Yield	Purity
	mg	mg	%	%
Concentrated urinary proteins ^b	5200 ^c	853	100	16
First Sephadex G-100 chromatography	1292 ^c	533	62	41
Zone electrophoresis	653 ^d	426	50	65
Second Sephadex G-100 chromatography	222 ^d	213	25	95
DEAE-Sephadex chromatography	85 ^d	83	10	98

^a Measured by single radial immunodiffusion.

^b The urinary proteins were obtained from two 24-h urine specimens from seven patients with chronic cadmium poisoning.

^c Determined by the Folin technique.

^d Estimated from the optical density at 280 nm.

Zone Electrophoresis in Barbital Buffer (pH 8.6)—The α_1 -microglobulin material from gel chromatography was separated by zone electrophoresis in 0.1 M barbital buffer, pH 8.6. Fig. 2 illustrates the distribution of total protein, α_1 -microglobulin, albumin, and transferrin from one of the zone electrophoretic experiments. The protein curve always showed peaks corresponding to the positions of α_1 -microglobulin and albumin. α_1 -Microglobulin was clearly heterogeneous in charge as it was distributed from the albumin to the transferrin region. Most of it apparently migrated in the slow α_1 -region. α_1 -Microglobulin from pooled and individual urines appeared to have similar charge heterogeneity. Proteins which migrated more slowly than transferrin were mainly free light chains of immunoglobulins (39). The eluates corresponding to the main part of the α_1 -microglobulin peak were combined and concentrated.

Second Gel Chromatography on Sephadex G-100—The α_1 -microglobulin fraction from zone electrophoresis was submitted to a second gel chromatography on Sephadex G-100. Fig. 3

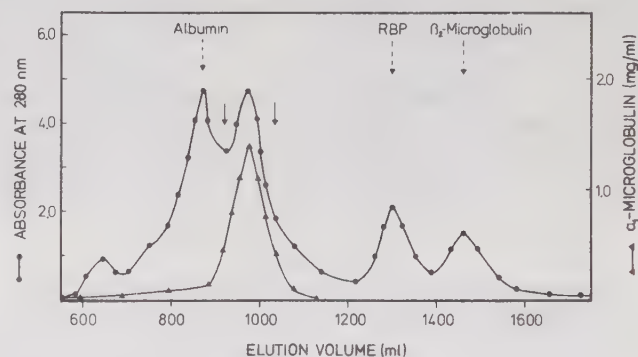


FIG. 1. First chromatography on Sephadex G-100 of concentrated urinary macromolecules from five patients with tubular proteinuria. The column (104 × 5 cm) was equilibrated with 0.02 M Tris/HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.02% NaN₃. The applied sample (10 ml) contained 1184 mg of total protein. Fractions of 9 ml were collected at a flow rate of 27 ml/h. The distribution in the effluent of α_1 -microglobulin was determined by single radial immunodiffusion. The peaks of albumin, retinol-binding protein (RBP), and of α_2 -microglobulin are also indicated. Fractions with α_1 -microglobulin were pooled as marked by the arrows.

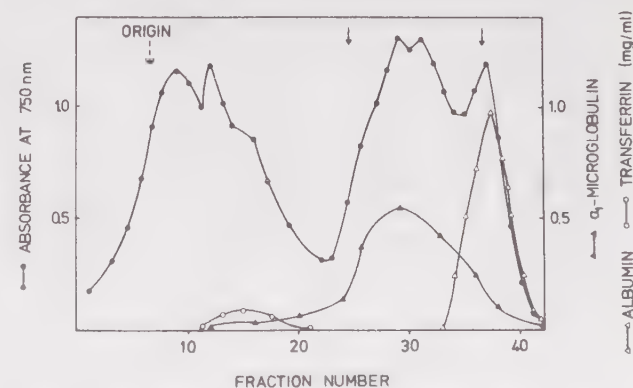


FIG. 2. Zone electrophoresis in 0.1 M barbital buffer, pH 8.6, of the α_1 -microglobulin-containing fraction obtained by chromatography on Sephadex G-100. The separated material (15 ml) contained 1176 mg of total protein. The potential gradient was 3.5 V/cm, and the time, 67 h. The distribution of total protein was measured by the Folin technique and the distribution of transferrin, albumin, and α_1 -microglobulin by single radial immunodiffusion. Fractions containing α_1 -microglobulin were combined as indicated by the arrows.

shows that at least three protein maxima were obtained. The main part of α_1 -microglobulin was present in the peak eluted last, but the small second peak apparently also was due to α_1 -microglobulin. The region between the two α_1 -microglobulin fractions contained albumin and orosomucoid, as shown by the single radial immunodiffusion technique. In some purifications, when the content of albumin and orosomucoid was relatively high, a third chromatography on Sephadex G-100 was carried out. The two α_1 -microglobulin fractions were concentrated separately.

Chromatography on DEAE-Sephadex-A-50—The main α_1 -microglobulin fraction from the second gel chromatography was dialyzed against 0.05 M Tris/HCl buffer, pH 7.5, containing 0.1 M NaCl and applied to a DEAE-Sephadex column which was equilibrated with the same buffer. The column was eluted with a linear salt gradient. Fig. 4 shows that α_1 -microglobulin was eluted as an asymmetrical peak, suggesting charge heterogeneity. Various fractions were examined by the Ouchterlony technique with the use of an antiserum against partially purified α_1 -microglobulin. No impurity was found in the main part of the α_1 -microglobulin peak. Traces of orosomucoid were detected by the single radial immunodiffusion method in a few tubes eluted early. The fractions were combined as indicated in Fig. 4, dialyzed exhaustively against distilled, deionized water, and lyophilized.

The purified α_1 -microglobulin had a brown color which could be observed during the entire isolation procedure.

Purity, Charge Heterogeneity, and Apparent Size Homogeneity

The purity of the isolated protein was assessed by immunoelectrophoresis and by Ouchterlony immunodiffusion analysis. Only a single precipitation line was observed when α_1 -microglobulin was tested against a polyvalent anti-urinary protein serum. A typical immunoelectrophoretic pattern is illustrated in Fig. 5. As can be seen from the figure, the antiserum used precipitates a large number of proteins in the starting material (urinary proteins from patients with chronic cadmium poisoning) but gives only one precipitation line with purified α_1 -microglobulin. α_1 -Microglobulin did not react with antisera against inter- α -trypsin-inhibitor, α_1 -antitrypsin, α_1 -B-glycoprotein, α_1 -T-glycoprotein, or α_1 -antichymotrypsin.

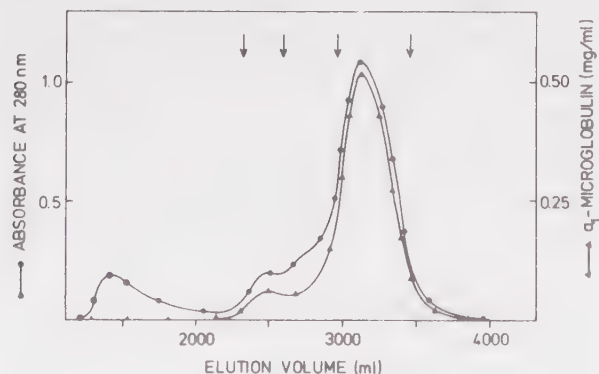


FIG. 3. Second chromatography on Sephadex G-100. The column (118 $\times$ 8 cm) was equilibrated with 0.02 M Tris/HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.02% NaN_3 . The sample (5.9 ml), obtained by zone electrophoresis in barbital buffer (Fig. 2), contained 262 mg of protein. Fractions of 20.0 ml were collected at a flow rate of 60 ml/h. Eluates corresponding to two peaks of α_1 -microglobulin, detected by single radial immunodiffusion, were pooled as indicated by the arrows.

The charge heterogeneity observed during the purification of α_1 -microglobulin was confirmed by submitting the protein to agarose gel electrophoresis and to polyacrylamide gel electrophoresis. Fig. 6 (left) shows a separation of concentrated urine from a patient with tubular proteinuria, purified α_1 -microglobulin, and normal serum by agarose gel electrophoresis. It is apparent that the α_1 -microglobulin band is relatively broad. Interpretation of the patterns of serum and urinary proteins on agarose gel electrophoresis has been done by Laurell (40). According to his classification the isolated main fraction of α_1 -microglobulin migrates in the α_1 -region and somewhat slower than serum α_1 -antitrypsin. In the pattern of urinary proteins there is a faint diffuse band with the mobility of purified α_1 -microglobulin.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate showed one narrow zone, suggesting size homogeneity (Fig. 7). Some preparations showed an additional faint staining at the leading edge of the zone.

Analyses of α_1 -microglobulin in the ultracentrifuge also indicated size homogeneity. Thus, sedimentation velocity analyses revealed a single component which remained symmetrical throughout the experiments. Furthermore, in sedi-

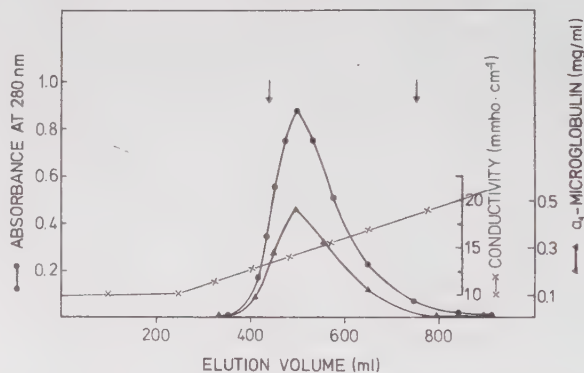


FIG. 4. Chromatography on DEAE-Sephadex A-50 of α_1 -microglobulin (117 mg) purified by chromatography on Sephadex G-100 (Fig. 3). The column (30 $\times$ 2.5 cm) was equilibrated with 0.05 M Tris/HCl buffer, pH 7.5, containing 0.1 M NaCl. Prior to application the sample was dialyzed exhaustively against the equilibrating buffer. Elution was performed with a 1000-ml linear gradient of sodium chloride from 0.1 to 0.3 M. Fractions of 12 ml were collected at 20-min intervals. The distribution of α_1 -microglobulin was determined by single radial immunodiffusion. The fractions were combined as marked by the arrows.



FIG. 5. Immunoelectrophoretic analyses of purified urinary α_1 -microglobulin and of starting material for the purification procedure. Buffer: 0.02 M sodium barbital, pH 8.6. The upper well contained purified α_1 -microglobulin and the lower well pooled concentrated urinary macromolecules from patients with chronic cadmium poisoning. An antiserum against urinary proteins of patients with tubular proteinuria was placed in the basin. The anode is to the left.

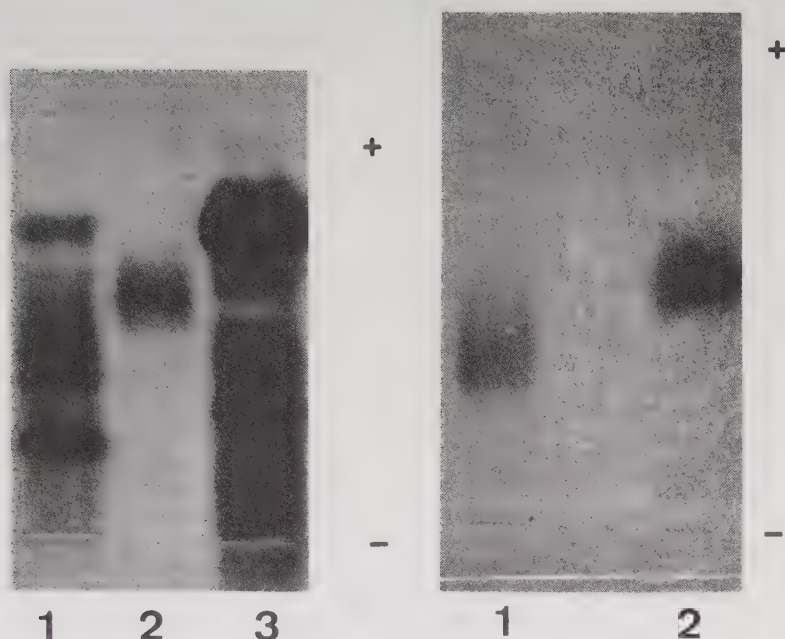


FIG. 6 (left). Agarose gel electrophoresis in 0.075 M barbital buffer, pH 8.6. *Left*, urinary proteins from a patient with tubular proteinuria (1), purified α_1 -microglobulin (2), and normal serum (3). *Right*, α_1 -microglobulin treated with neuraminidase (1) and untreated α_1 -microglobulin (2). The anode is at the top.

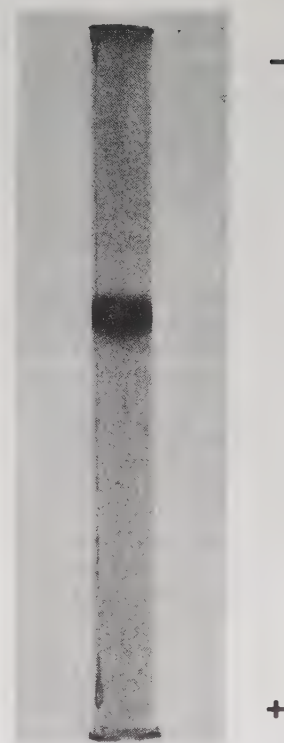


FIG. 7 (right). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified α_1 -microglobulin. The pH in the running gel was 9.2. An acrylamide concentration of 13.7% was used. Electrophoresis proceeded from top to bottom.

mentation equilibrium experiments the plot of the logarithm of protein concentration *versus* the square of the distance to the axis of rotation was linear.

Formation of Dimers

When partially purified or purified α_1 -microglobulin was submitted to gel chromatography a small fraction eluted early was always seen in addition to the main fraction (Fig. 3). This observation indicated that α_1 -microglobulin has a tendency to form dimers or higher polymers. The molecular weights of the two fractions were compared by gel chromatography according to the method of Andrews (17). With this method the α_1 -microglobulin fractions had apparent molecular weights of about 80,000 and 40,000. Accordingly, the larger material appears to be composed of dimers of the main α_1 -microglobulin species, assuming that both species have similar shapes. It should be noted that the molecular weight values obtained with the method of Andrews are considerably higher than the values obtained with other methods (see below).

On polyacrylamide gel electrophoresis in sodium dodecyl sulfate and on starch gel electrophoresis in 8 M urea at acid pH, both the unreduced and reduced dimeric form of α_1 -microglobulin had the same mobility as the monomeric form. This indicates that the dimers are noncovalently linked.

Other Properties of α_1 -Microglobulin

Various physical and chemical properties of α_1 -microglobu-

lin are given in Tables II and III. It is apparent that this protein is a relatively small glycoprotein.

The same sedimentation coefficient, 2.35 S, was found for two α_1 -microglobulin preparations. It was independent of protein concentrations, ranging from 1.2 to 6.2 mg/ml. Four determinations of Stokes molecular radius gave values ranging from 2.81 to 2.88 nm.

The molecular weight was estimated by sedimentation equilibrium ultracentrifugation on three preparations of α_1 -microglobulin. The values obtained were 27,000, 27,000, and 26,200, at protein concentrations of 1.7, 1.2, and 0.6 mg/ml, respectively. Molecular weight measurements on three samples of the protein by gel chromatography in 6 M guanidine HCl gave the values 24,000, 25,000, and 25,500. The molecular weights obtained by these two methods and by calculation from the sedimentation and diffusion coefficient were quite similar (Table II). The considerably higher values of 30,000 to 32,000 (mean 31,000) were found in ten experiments with polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

Amino acid and carbohydrate analyses were performed on three preparations of α_1 -microglobulin and are presented in Table III. The number of residues was calculated on the basis of 11.0 residues of leucine per molecule and the carbohydrate content given in Table II. The nearest integer values give a calculated molecular weight of 26,100.

The carbohydrate portion of the protein amounted to 20% of its weight and consisted of glucosamine, mannose, galactose, and sialic acid (Tables II and III). Traces of galactosamine and fucose were also observed. The values of glucosamine

Human α_1 -Microglobulin. Isolation and Properties

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TABLE II

Physical and chemical properties of human α_1 -microglobulin

Sedimentation coefficient, $s_{20,w}^0$	2.35 S
Stokes molecular radius, r_s	2.85 nm
Partial specific volume, $\bar{V}$ (calculated) ^a	0.704 ml/g
Apparent diffusion coefficient, $D_{20,w}$	75 $\mu\text{m}^2 \text{s}^{-1}$
Molecular weight	
By sedimentation equilibrium ultracentrifugation	26,700
From sedimentation and diffusion coefficients	26,100
By gel chromatography in 6 M guanidine HCl after reduction and alkylation	24,800
By electrophoresis in sodium dodecyl sulfate	31,000
Frictional ratio, f/f_0	1.45
Absorption coefficient at 280 nm (pH 7.0)	$4.72 \cdot 10^4 \text{ M}^{-1} \text{ cm}^{-1}$
Mean residue ellipticity at 216 nm	-1900 deg cm^2/dmol
Nitrogen content	
Experimental	12.3%
Calculated	13.2%
Free sulfhydryl groups	Nil
Carbohydrate	19.5%
Glucosamine ^b	5.6%
Mannose	4.3%
Galactose	3.8%
Sialic acid ^c	5.8%

^a From amino acid and carbohydrate composition.^b Determined by gas-liquid chromatography-mass spectrometry and calculated as *N*-acetyl derivative. The glucosamine content measured in the amino acid analyzer was 5.1%.^c Measured by the Bial assay. The value obtained with the thiobarbituric acid method was 5.1%.

obtained by two separate techniques were in good agreement. This was also true for the sialic acid values obtained by two techniques (Table II). The content of neutral sugars (mannose + galactose) was 8.1% as determined by gas-liquid chromatography-mass spectrometry and 6.4% as determined by the orcinol method. As shown in Table III, the four monosaccharides are present in close to equimolar amounts.

After digestions of α_1 -microglobulin with neuraminidase, up to 94% of the sialic acid was removed. On agarose gel electrophoresis, neuraminidase-treated and untreated α_1 -microglobulin showed similar electrophoretic heterogeneity but the neuraminidase-treated protein had a lower mobility (Fig. 6, right).

The recovery of amino acids in the amino acid analyses corresponded to about 75% of the weight of the protein. As the carbohydrate content was about 20%, α_1 -microglobulin could possibly contain about 5% of unspecified constituents. The theoretical nitrogen content, calculated from the amino acid and carbohydrate composition, was somewhat higher than the experimental value (Table II). The discrepancy between the experimental and calculated nitrogen values could also indicate the presence in α_1 -microglobulin of a small amount of constituents other than those determined.

No free sulfhydryl groups were detected by titration with 5,5'-dithiobis(2-nitrobenzoic acid) or by alkylation with iodo[¹⁴C]acetamide in 6 M guanidine hydrochloride. Determination of half-cystine as cysteic acid after performic acid oxidation indicated the presence of 3.4 residues per molecule. When half-cystine was determined as carboxymethylcysteine after reduction and alkylation in 6 M guanidine hydrochloride followed by extensive dialysis first against the same solvent

TABLE III

Amino acid and carbohydrate composition of human α_1 -microglobulin

Except where noted, all figures are averages of values from one 24-h hydrolysate and one 72-h hydrolysate. Three preparations (I, II, and III) were analyzed.

Constituent	Found			To nearest integer
	Preparation I	Preparation II	Preparation III	
	residues/molecule ^a			
Aspartic acid	14.1	14.1	14.6	14
Threonine ^b	17.2	16.6	17.0	17
Serine ^b	10.4	10.1	10.3	10
Glutamic acid	21.4	21.6	22.2	22
Proline	14.0	13.5	12.9	13
Glycine	14.0	13.8	14.2	14
Alanine	9.1	9.2	9.6	9
Half-cystine ^c	3.4	3.4		3
Valine ^d	10.7	10.7	11.2	11
Methionine	4.4	4.5	4.6	5
Isoleucine	12.7	12.7	11.9	12
Leucine	11.0	11.0	11.0	11
Tyrosine	8.0	8.4	8.4	8
Phenylalanine	6.1	6.1	6.4	6
Lysine	10.3	10.6	10.9	11
Histidine	3.5	3.7	3.7	4
Arginine	9.8	9.6	9.6	10
Tryptophan ^e	2.7			3
Glucosamine	7.2 ^f	6.7 ^g	5.7 ^g	7
Mannose ^f	6.9			7
Galactose ^f	6.2			6
Sialic acid ^h	5.5	5.0	5.1	5
Total				208

^a Calculated on the basis of 11.0 residues of leucine per molecule.^b Values obtained by extrapolation to zero hours hydrolysis.^c Determined as cysteic acid after performic acid oxidation.^d Seventy-two-hour hydrolysis value only.^e Tryptophan was estimated spectrophotometrically. Another preparation of α_1 -microglobulin gave a value of 3.0.^f Determined as alditol acetates by gas-liquid chromatography-mass spectrometry.^g Measured in the amino acid analyzer.^h Estimated by the Bial reaction.

and then against deionized water, the value was estimated at 2.3.

Starch gel electrophoresis in 8 M urea and formate buffer, pH 3.0, of α_1 -microglobulin and of α_1 -microglobulin treated with urea revealed only one broad protein zone. α_1 -Microglobulin reduced and alkylated in the presence of urea had the same electrophoretic mobility as the unreduced protein.

The absorption curve of α_1 -microglobulin at pH 7.0 showed a maximum at 278 nm, an inflexion point at 289 nm and an absorption which decreased slowly from about 320 nm throughout the ultraviolet and visible ranges. The spectrum from 239 to 450 nm is shown in Fig. 8. The absorbances at 500 and 700 nm were 0.05 and 0.04, respectively. The molar extinction coefficient at 280 nm was determined at $4.72 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. Calculations based on the amino acid composition according to the method of Wetlaufer (41) gave the considerably lower value of $2.81 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. The high absorption at 280 nm and the absorption in the near-ultraviolet and visible ranges most likely is due to material(s) which give α_1 -microglobulin its brown color.

Attempts were made to eliminate the colored material(s) from α_1 -microglobulin. Dissociation of these material(s) could

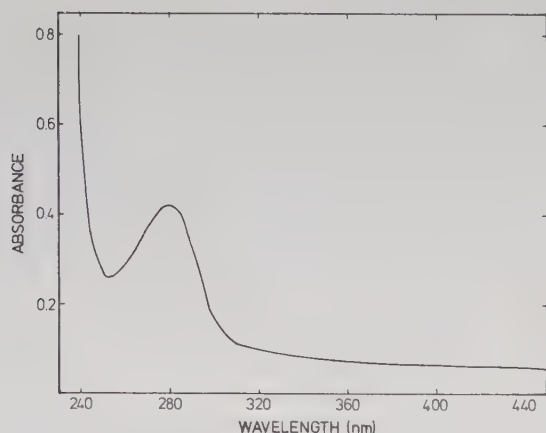


FIG. 8. Ultraviolet absorption spectrum of α_1 -microglobulin. The protein (0.3 mg/ml) was dissolved in 0.1 M sodium phosphate buffer, pH 7.0.

not be achieved by gel chromatography of untreated α_1 -microglobulin on Sephadex G-100 in 1 M acetic acid, or by chromatography of reduced and alkylated protein on Sepharose CL-6B in 6 M guanidine hydrochloride.

The CD spectrum of α_1 -microglobulin in the range of 200 to 350 nm is given in Fig. 9, A and B. Each curve represents the average of measurements on two different samples. The most prominent feature of the spectrum is a negative band at 216 nm with a magnitude of $-1900 \text{ deg cm}^2/\text{dmol}$. Above 230 nm the ellipticity is low. There are bands located at 253(+), 264(+), 268(-), 273(-), 283(-), and 291(-). In the range of 300 to 350 nm there is a positive band at 310 nm.

Demonstration of α_1 -Microglobulin in Human Biological Fluids

The occurrence of α_1 -microglobulin in normal serum and urine was established by Ouchterlony immunodiffusion analyses. Fig. 10 shows that material reacting like α_1 -microglobulin is present in these two biological fluids. The α_1 -microglobulin from the different sources gave a reaction of immunological identity. Five samples of cerebrospinal fluid contained only traces of α_1 -microglobulin. On immunoelectrophoresis, α_1 -microglobulin in concentrated individual urines from healthy persons and from patients with tubular proteinuria seemed to have the same electrophoretic mobility.

Concentrated normal urine was separated on Sephadex G-100 and G-200 columns equilibrated with 0.02 M Tris/HCl buffer, pH 8.0, containing 0.15 M NaCl. Analyses of the effluent by single radial immunodiffusion showed a small and a large peak of material that reacted with anti- α_1 -microglobulin serum; some material related to α_1 -microglobulin was found also between the two peaks. The small peak emerged at the void volume of the column and the large peak had the same elution volume as purified α_1 -microglobulin.

Gel chromatography of fresh and stored plasma or serum on Sephadex G-200 revealed similar patterns with four fractions containing α_1 -microglobulin (4). The smallest plasma component corresponded in molecular size to α_1 -microglobulin purified from urine. In immunoelectrophoretic experiments, the smallest plasma component and urinary α_1 -microglobulin appeared to have the same electrophoretic mobility. The two proteins also gave a reaction of immunological identity on Ouchterlony immunodiffusion analyses.

The quantities of α_1 -microglobulin in serum and urine from healthy individuals and from patients with renal diseases

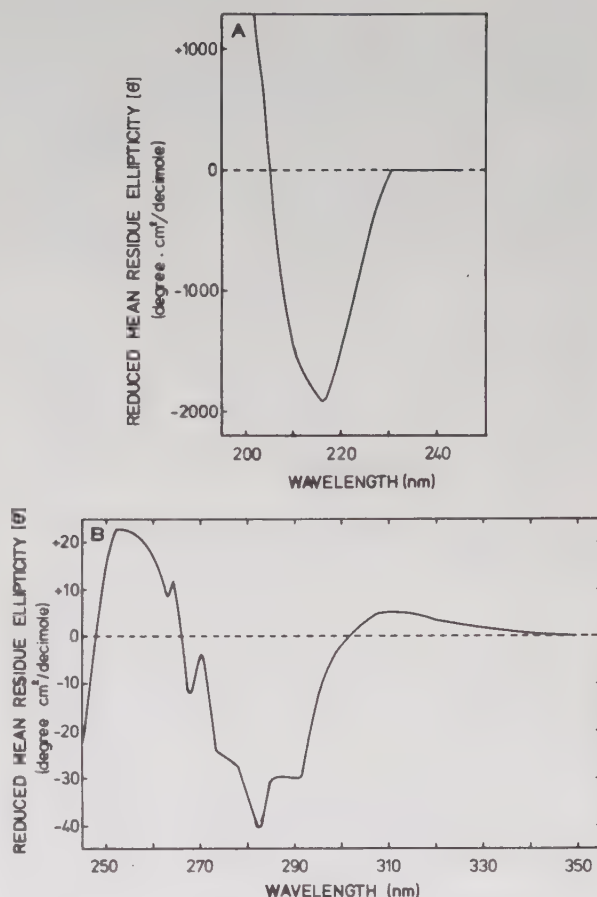


FIG. 9. Circular dichroism spectra of α_1 -microglobulin. A, between 200 and 245 nm; B, between 245 and 350 nm.

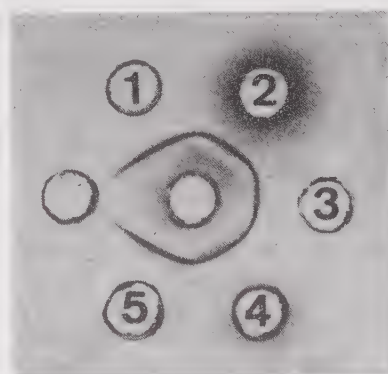


FIG. 10. Ouchterlony immunodiffusion analysis of purified α_1 -microglobulin (1, 3, and 5), normal serum (2), and concentrated normal urine (4). The center well contained anti- α_1 -microglobulin serum.

were determined by the single radial immunodiffusion technique. Unconcentrated samples were used for these analyses. The results given in Table IV show that the concentration of α_1 -microglobulin is higher in normal serum than in normal urine. The α_1 -microglobulin concentration in umbilical cord blood serum was about half of that in normal serum. Both serum and urine from patients with renal diseases contained much higher amounts of α_1 -microglobulin than serum and urine from healthy persons. Accurate figures for the content of α_1 -microglobulin in serum can not be given at the present

TABLE IV

Quantities of human α_1 -microglobulin in serum and urine from normal individuals and from patients with renal diseases

Fluid	Content ^a	
	Mean	Range
Serum from healthy individuals (mg/l)	54	34-90
Serum of umbilical cord blood (mg/l)	26	18-36
Serum from patients with renal failure (mg/l)	298	210-385
Urine from healthy individuals (mg/24-h volume)	9 ^b	6-15
Urine from patients with chronic cadmium poisoning (mg/24-h volume)	182	110-250

^a Determined by a single radial immunodiffusion technique. Measurements for each fluid were carried out on samples from 10 individuals.

^b The mean content per liter of urine was 8 mg (range 6 to 11 mg).

time because serum contains several components that react with anti- α_1 -microglobulin sera (see above).

DISCUSSION

α_1 -Microglobulin has two properties of particular interest. It contains prosthetic group(s) that give the protein a brown color and it is heterogeneous in charge. The chromophore material is strongly linked to the protein as it was not removed by reduction and alkylation, or by treatment with 1 M acetic acid or 6 M guanidine hydrochloride. α_1 -Microglobulin absorbs ultraviolet and visible light in a broad wavelength range, suggesting that the chromophore material is not homogeneous.

On electrophoresis and ion-exchange chromatography, the protein purified from both pooled and individual urines showed charge heterogeneity. This heterogeneity was in contrast to the apparent size homogeneity demonstrated by analytical ultracentrifugations and by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. To determine whether the charge heterogeneity depends on a varying number of sialic acid residues, digestion with neuraminidase was performed. On agarose gel electrophoresis the neuraminidase-treated α_1 -microglobulin had a reduced mobility, but showed a similar charge heterogeneity as the untreated protein. This suggests that the heterogeneity resides in the polypeptide chain or in the chromophore material. Frangione *et al.* (7) have found a unique sequence for the first 25 amino acids from the NH_2 -terminal end of α_1 -microglobulin (protein HC) purified from a single individual. Therefore, at least the intraindividual charge heterogeneity is apparently not due to polymorphism of the NH_2 -terminal part of the molecule.

We have found that serum or plasma from healthy individuals contains a minimum of four components that react with antisera against α_1 -microglobulin and that differ in size (4). The smallest serum component and urinary α_1 -microglobulin appear to have the same size, antigenic structure, and electrophoretic mobility and are probably identical. Tejler and Grubb (5) have presented evidence for the occurrence in serum of complexes of α_1 -microglobulin (protein HC) with albumin and with IgA. Two of the serum components observed by us may correspond to such complexes. Other components could possibly be oligomers or polymers of α_1 -microglobulin.

α_1 -Microglobulin apparently is made up of a single polypeptide chain because approximately the same molecular weight was estimated by analytical gel chromatography of the reduced and alkylated protein in 6 M guanidine hydrochloride

and by analytical ultracentrifugations of the native protein in the absence of a dissociating agent. On gel chromatography in a close to physiological salt solution, purified α_1 -microglobulin had some tendency to form dimers. Electrophoretic analyses in dissociating media indicated that the dimers are noncovalently linked. As shown by gel chromatography, concentrated urines contain small amounts of big molecules that react with antisera against α_1 -microglobulin. These big molecules might be polymers of α_1 -microglobulin or complexes of α_1 -microglobulin with other proteins (see above).

The amino acid composition of our preparations of α_1 -microglobulin is similar to the figures published by Frangione *et al.* (7) and Svensson and Ravnskov (8). But our and their analyses are not entirely comparable because Frangione *et al.* (7) have examined the protein only after 20 h hydrolysis, and Svensson and Ravnskov (8) have analyzed only unoxidized protein. The composition reported by Tejler and Grubb (5) differs from the composition given in this paper with respect to the content of several amino acids, such as serine, alanine, half-cystine, isoleucine, and lysine. None of the other groups have determined the content of tryptophan.

We have previously given a preliminary figure of 23% for the carbohydrate content of α_1 -microglobulin (4). This preliminary figure was based solely on the results of colorimetric analyses. By using gas-liquid chromatography-mass spectrometry, and the amino acid analyzer, we have now found that α_1 -microglobulin contains close to equimolar amounts of glucosamine, mannose, galactose, and sialic acid, with a total carbohydrate content of about 20%. The previously determined hexosamine value apparently was too high, probably due to interference in the colorimetric assay by the chromophore part of the molecule. The carbohydrate content given here agrees fairly well with that reported by Svensson and Ravnskov (8), but is lower than the content found by Tejler and Grubb (5). The latter authors also found appreciable amounts of galactosamine. They purified α_1 -microglobulin (protein HC) from normal urine, which contains relatively small amounts of this protein compared to various other carbohydrate-rich macromolecules (9). The somewhat divergent carbohydrate and amino acid composition of Tejler and Grubb's preparation might be due to some contamination by such other macromolecules.

α_1 -Microglobulin apparently is devoid of free sulfhydryl groups. The number of half-cystine residues was about 3.4 per molecule when measured as cysteic acid after performic acid oxidation and about 2.3 when measured as carboxymethylcysteine after reduction, alkylation, and dialysis in 6 M guanidine hydrochloride. This suggests that α_1 -microglobulin contains at least 2 half-cystine residues which could either be blocked by, *e.g.* cysteine, or be involved in a disulfide bond. The former possibility is supported by the lower value obtained after extensive reduction and dialysis. On urea-starch gel electrophoresis, the mobility of a protein is often decreased after reduction of an intrachain disulfide bridge (2). No such decreased mobility was observed after extensive reduction of α_1 -microglobulin. This suggests the absence of a disulfide loop or the presence of only a small loop.

The molecular weight of about 26,000 estimated by ultracentrifugation or by gel chromatography in 6 M guanidine hydrochloride is in contrast to the value of about 31,000 measured by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Other groups have reported a molecular weight of 31,000 based only on analyses in sodium dodecyl sulfate polyacrylamide gels (5, 8). α_1 -Microglobulin contains carbohydrate and

seems to have an elongated shape (see below). Both properties could contribute to overestimation of the molecular weight upon sodium dodecyl sulfate gel electrophoresis (42, 43).

The frictional ratio of α_1 -microglobulin is relatively high (1.45). Typical globular and carbohydrate-free proteins have frictional ratios ranging from 1.1 to 1.3 (44). A protein with a high frictional ratio may be highly asymmetric, or highly solvated, or both. α_1 -Microglobulin may have a relatively high solvation due to its carbohydrate portion but it is unlikely that this is the only reason for its high frictional ratio. Thus, α_1 -acid glycoprotein which has a similar frictional ratio (1.50) is both very rich in carbohydrate (40%) and asymmetric (45). It seems probable that α_1 -microglobulin, too, has an elongated shape.

CD spectra of the protein were dominated by a negative band with a minimum at 216 nm, close to the minimum (217 nm) reported to be characteristic for the β -pleated sheet conformation (23, 46). Accordingly, α_1 -microglobulin appears to contain β structures, but the CD analyses did not give any indication for the presence of α helical structure. It is difficult to identify the various small CD bands observed in the 245 to 350 nm region. Phenylalanine, tyrosine, tryptophan, or cystine residues, or all of these, in asymmetric environments are probably mainly responsible for these bands (46). But the chromophore material(s) in α_1 -microglobulin might also contribute.

It is difficult to obtain reliable estimates of the concentration of α_1 -microglobulin in various biological fluids because this protein occurs in forms with higher molecular weight both in serum (4) and in urine and because it is heterogeneous in charge. Therefore, it is not surprising that our figure for the mean level of α_1 -microglobulin in normal serum (54 mg/liter) differs from the figures reported by Tejler and Grubb (5) (98 mg/liter) and by Svensson and Ravnskov (8) (32 mg/liter). We have determined the protein by single radial immunodiffusion, whereas the two other groups have used electroimmunoassay. Our present figures for the urinary excretion of α_1 -microglobulin are similar to those reported by Tejler and Grubb (5) but higher than our preliminary values (4) and the values found by Svensson and Ravnskov (8). One possible explanation for the discrepancy is that the present analyses were made on samples of fresh unconcentrated urine, whereas our previous analyses were made on concentrated urines that may have contained aggregated α_1 -microglobulin. The possibility of some degradation of α_1 -microglobulin in concentrated and stored samples may also have to be considered.

Information about the origin of α_1 -microglobulin is still very limited. Tejler *et al.* (6) have reported that it is present on the surface of the majority of normal human peripheral lymphocytes. α_1 -Microglobulin may not be synthesized in the liver because Svensson and Ravnskov found normal serum levels in patients with severe reduction of plasma protein synthesis caused by cirrhosis of the liver (8). We have recently isolated α_1 -microglobulin from the guinea pig¹ and hope that studies on this animal will shed more light on the origin and function of α_1 -microglobulin.

Like other plasma proteins in the molecular weight range below 50,000, α_1 -microglobulin is eliminated from the blood probably mainly in the kidneys, by glomerular filtration followed by tubular reabsorption and catabolism (1). A renal elimination of α_1 -microglobulin is supported by the increased levels of the protein in the blood of patients with renal failure and in the urine of patients with tubular disease.

¹ B. Åkerström and I. Berggård, unpublished work.

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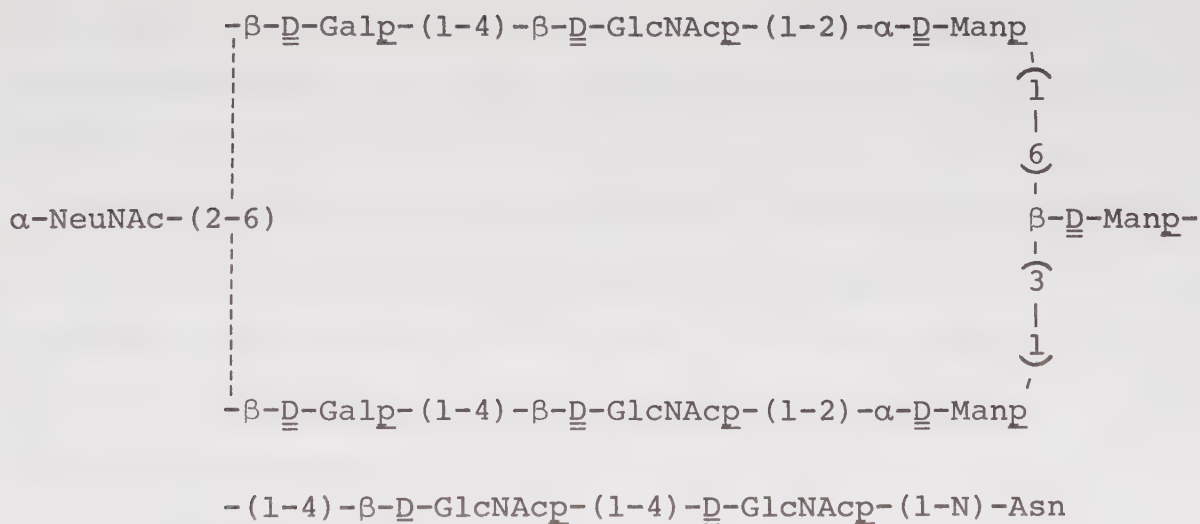
STRUCTURAL STUDIES ON THE CARBOHYDRATE PORTION
OF HUMAN α_1 -MICROGLOBULIN

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Summary

Human α_1 -microglobulin has been shown to have three N-glycosidically linked carbohydrate chains of the following structure:



This structure was established by sugar and methylation analysis before and after removal of the N-acetylneuraminic acid residue and by using specific chemical degradation techniques such as Smith degradation, chromium trioxide oxidation and trifluoroacetylation.

Introduction

Human α_1 -microglobulin is present in serum and in urine of healthy individuals. The urine from patients with chronic cadmium poisoning contains α_1 -microglobulin in elevated amounts (1).

α_1 -Microglobulin is a brown-coloured glycoprotein with a molecular weight of about 26,000. It contains about 17% carbohydrate including the monosaccharide units NeuNAc¹, D-Gal, D-Man, and D-GlcNAc (1-4). The biological function of α_1 -microglobulin is not known.

The present investigation deals with structural studies on the carbohydrate portion of α_1 -microglobulin.

Materials and Methods

Analytical Methods. - Colorimetric methods were used for the determination of total hexose (5) and N-acetylneuraminic acid (6). Sodium ion was determined by flame photometry. Sugar analysis was performed, after hydrolysis with 90% aqueous formic acid at 100°C for 5 h followed by hydrolysis with 0.25 M aqueous sulphuric acid at 100°C for 18 h, by gas-liquid chromatography (7) and mass spectrometry (8). Methylation analysis was performed as described previously (9). Gas-liquid chromatography was carried out on a Perkin-Elmer 3920 gas chromatograph

¹ The abbreviations used are: NeuNAc, N-acetylneuraminic acid; Galp, galactopyranose; GlcNAcp, 2-acetamido-2-deoxy-glucopyranose; GlcNTF, 2-deoxy-2-trifluoroacetamido-glucopyranose; Manp, mannopyranose; Man-OL, mannitol; TFA, trifluoroacetic acid; TFAA, trifluoroacetic anhydride.

equipped with a flame ionisation detector. Separations were performed on (a) an SE-30 W.C.O.T. glass capillary column (50 m x 0.25 mm) at 190°C (for partially methylated alditol acetates) and at 180°C-330°C (for permethylated oligosaccharide alditols), (b) a glass column (2 m x 0.5 cm) packed with 3% ECNSS-M on Chromosorb Q at 200°C (for alditol acetates). Gas-liquid chromatography-mass spectrometry was performed on a Varian MAT 311A instrument fitted with the appropriate column. The spectra were recorded at 70 eV, with an ionisation current of 3 mA and an ion source temperature of 120°C. The spectra were processed on an on-line computer system (Spectrosystem 100, Varian MAT).

Human α_1 -Microglobulin. - This was obtained from patients with tubular or mixed glomerular-tubular proteinuria (2). The α_1 -microglobulin preparation was homogeneous in size as judged by polyacrylamide gel electrophoresis and by ultracentrifugation (2).

Preparation of Asialo α_1 -Microglobulin. - This was obtained by treatment of α_1 -microglobulin with Vibrio cholerae neuraminidase (Behringwerke AG, Marburg/Lahn, West Germany) (2) or by mild acid hydrolysis (10). Both procedures removed more than 95% of the NeuNAc residues.

Trifluoroacetolysis of α_1 -Microglobulin. - Freeze-dried α_1 -microglobulin (115 mg) was added to a solution of TFA/TFAA (30 ml, 1:1, v/v) and the reaction mixture was sealed in a glass vessel. The reaction mixture was heated at 100°C for 48 h (Caution! Corrosive mixture under pressure!). The resulting clear, dark solution was cooled and evaporated to dryness. The product was dissolved in methanol (25 ml) and the solution was

evaporated to dryness. The residue was then treated at room temperature with 50% aqueous acetic acid (25 ml) and after 30 min at 100°C the reaction mixture was evaporated to dryness. The product was partitioned between diethyl ether (50 ml) and water (25 ml). The ethereal layer was extracted twice with water (25 ml). The combined water phases were extracted once with diethyl ether (25 ml) and concentrated to 10 ml. To the water solution was added sodium borodeuteride (100 mg) and after 18 h the reduction mixture was acidified with glacial acetic acid and concentrated to dryness. Boric acid was removed by three codistillations with methanol (10 ml) and the resulting product was desalted on a Sephadex G-25 column (3 x 40 cm) eluted with distilled water. The desalted product was acetylated with acetic anhydride/pyridine (1:1, v/v, 10 ml) at 100°C for 1 h. After evaporating the reagents the acetylated product was de-O-acetylated by treatment with 1 M ammonia in 75% aqueous methanol (25 ml) at room temperature for 18 h. The reaction mixture was evaporated to dryness and fractionated on a Sephadex G-25 column (1.6 x 40 cm) eluted with distilled water. One carbohydrate-containing peak was obtained which was pooled and lyophilized to yield 4 mg of product.

Chromium Trioxide Oxidation of the Oligosaccharide

Obtained after Trifluoroacetolysis of α_1 -Microglobulin. - To about 3 mg of acetylated oligosaccharide obtained by trifluoroacetolysis of α_1 -microglobulin (see above) perseitol heptaacetate was added as internal standard and the mixture was divided into two portions. One portion was subjected to sugar analysis and the other portion was dissolved in glacial acetic acid (2 ml). Powdered chromium trioxide (20 mg) was added to

the acetic acid solution and the resulting suspension was agitated in an ultrasonic bath at 50°C for 2 h. The suspension was then cooled, diluted with water (50 ml) and the resulting solution was extracted three times with chloroform (10 ml). The combined chloroform phases were washed with water (2 x 10 ml) and evaporated to dryness. The oxidized product was subjected to sugar analysis and investigated by gas-liquid chromatography-mass spectrometry after permethylation (11,12).

Smith Degradation - Trifluoroacetolysis of α_1 -Microglobulin. - α_1 -Microglobulin (300 mg) was dissolved in sodium acetate buffer (0.1 M, pH 4.0) (50 ml) and 170 mg of sodium metaperiodate dissolved in sodium acetate buffer (0.1 M, pH 4.0) (50 ml) was added to this solution. The periodate oxidation was carried out at 4°C in the dark. After 48 h excess of periodate was destroyed by the addition of ethylene glycol (1 ml). Sodium borohydride, 250 mg, was added in small portions and the reduction was carried out at 4°C for 20 h. The reduction mixture was acidified (pH 3) by the addition of glacial acetic acid. The solution was concentrated to dryness and boric acid was removed by codistillation with methanol (3 x 10 ml). The resulting product was desalted by dialysis. The yield of periodate-oxidized and reduced material was 190 mg. Part of this material was subjected to sugar and methylation analysis.

Periodate-oxidized and reduced material (185 mg) was hydrolysed in 0.1 M aqueous hydrochloric acid (40 ml) for 1 h at 80°C. The hydrolysis mixture was cooled, neutralised with sodium hydrogen carbonate and dialyzed extensively against distilled water. The dialyzed material was lyophilized and subjected to sugar and methylation analysis. The contents of

the concentrated dialysis water was reduced with sodium borodeuteride and acetylated. The acetylated product was partitioned between chloroform and water and the chloroform phase was washed with water. The acetylated product was then permethylated and analyzed by gas-liquid chromatography-mass spectrometry. A single oligosaccharide peak was obtained with a mass spectrum and gas-liquid chromatographic retention time identical to that of β -D-GlcNAc $\underline{p}$ -(1-2)-[1-D]-glycerol (12).

The non-dialyzable fraction (160 mg) was subjected to trifluoroacetolysis in TFA/TFAA (30 ml, 1:50, v/v) for 48 h at 100°C. The reaction mixture was worked up as described above for trifluoroacetolysis of α_1 -microglobulin. The product (5.0 mg) was fractionated on a Sephadex G-25 column (1.5 x 80 cm) (Fig. 1) to yield two oligosaccharide peaks which were subjected to sugar and methylation analysis.

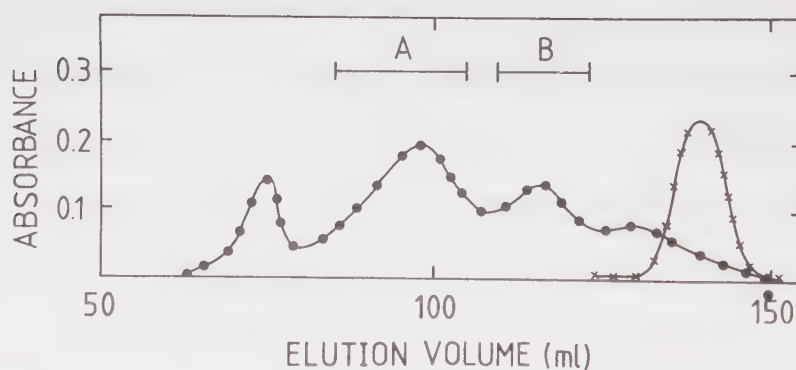


Fig. 1. Fractionation on a Sephadex G-25 column (1.5 x 80 cm) of the non-dialyzable product (5 mg) from Smith degradation-trifluoroacetolysis of α_1 -microglobulin. Eluted with water. 5 ml fractions, flow rate 10 ml/h. ●—● Total hexose. X—X Sodium ion. Fractions were pooled as indicated in the figure.

Results and Discussion

Sugar analysis of human α_1 -microglobulin showed $\underline{\underline{D}}$ -Man (4.9%), $\underline{\underline{D}}$ -Gal (3.8%), $\underline{\underline{D}}$ -GlcNAc (5.8%), and NeuNAc (2.5%) corresponding to the relative molar proportions 3.0:2.2:3.7:1.1. Methylation analysis of α_1 -microglobulin before and after desialylation (Table I) demonstrated the following structural elements in the original carbohydrate portion:

NeuNAc-(2-6)- $\underline{\underline{D}}$ -Galp-(1-	1 residue
-4)- $\underline{\underline{D}}$ -GlcNAcp-(1-	4 residues
-2)- $\underline{\underline{D}}$ -Manp-(1-	2 residues
-3)- $\underline{\underline{D}}$ -Manp-(1-	1 residue
 6 	
$\underline{\underline{D}}$ -Galp-(1-	1 residue

TABLE I. Methyl ethers obtained from hydrolysates of methylated α_1 -microglobulin and desialylated α_1 -microglobulin

Sugars	T-value ^a SE-30	Relative molar proportions	
		α_1 -Micro- globulin	α_1 -Micro _b - globulin
2,3,4,6-O-Me- $\underline{\underline{D}}$ -Gal	1.07	1.0 (1) ^c	2.1 (2)
2,3,4-O-Me- $\underline{\underline{D}}$ -Gal	1.82	0.7 (1)	<0.05 (0)
3,4,6-O-Me- $\underline{\underline{D}}$ -Man	1.45	1.8 (2)	2.2 (2)
2,4-O-Me- $\underline{\underline{D}}$ -Man	2.40	0.8 (1)	0.8 (1)
3,6-O-Me- $\underline{\underline{D}}$ -GlcN(Me)Ac	4.15	) + (4) ^d	+ (4) ^d
3,6-O-Me- $\underline{\underline{D}}$ -GlcNAc	4.32		

^a Retention times of the corresponding alditol acetates relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl- $\underline{\underline{D}}$ -glucitol.

^b Desialylated.

^c Figures in parentheses represent nearest integer.

^d The relative molar proportions were calculated from the sugar analysis (see text).

In order to determine the sequential arrangement and anomeric configurations of the sugar units in α_1 -microglobulin it was subjected to trifluoroacetylytic degradation (13-18) under conditions (TFA/TFAA, 1:1, 100°C, 48 h) that will cleave off the carbohydrate chains from the protein moiety, cleave off 6-linked NeuNAc residues, degrade reducing $\underline{\underline{D}}$ -GlcNAc residues and transamidate $\underline{\underline{D}}$ -GlcNAc units into $\underline{\underline{D}}$ -GlcNTF residues. The trifluoroacetylysis also degraded the protein part by transamidation. After de-O- and de-N-trifluoroacetylation by reduction with sodium borodeuteride followed by N-acetylation a reconstituted oligosaccharide was obtained. Sugar analysis showed $\underline{\underline{D}}$ -Gal, $\underline{\underline{D}}$ -Man, $\underline{\underline{D}}$ -GlcNAc and $\underline{\underline{D}}$ -[1-D]-Man-OL in the relative proportions 2.0:1.9:2.1:1.0 respectively. Methylation analysis gave 2,3,4,6-tetra-O-methyl- $\underline{\underline{D}}$ -Gal, 3,4,6-tri-O-methyl- $\underline{\underline{D}}$ -Man, 3,6-di-O-methyl- $\underline{\underline{D}}$ -GlcNAc (and the N-methylated derivative) and 1,2,4,5-tetra-O-methyl- $\underline{\underline{D}}$ -[1-D]-Man-OL in the relative molar proportions 2.0:1.8:2.1:0.8 respectively. The oligosaccharide was acetylated and subjected to chromium trioxide oxidation in acetic acid (19). Chromium trioxide in acetic acid will oxidize glycosides, with the aglycone equatorially oriented (β -linked), into 5-hexulosonates whereas glycosides with the aglycone axially oriented (α -linked) are unaffected. Sugar analysis of the acetylated, oxidized oligosaccharide showed $\underline{\underline{D}}$ -Man and $\underline{\underline{D}}$ -[1-D]-Man-OL in the relative molar proportions 2:1 together with only traces of $\underline{\underline{D}}$ -Gal and $\underline{\underline{D}}$ -GlcNAc, demonstrating that the $\underline{\underline{D}}$ -Gal and $\underline{\underline{D}}$ -GlcNAc residues were β -linked and the $\underline{\underline{D}}$ -Man residues are α -linked. On treatment of the acetylated, oxidized oligosaccharide with base, the 5-hexulosonate linkages are broken, liberating the aglycones. Permethylation of the

dialyzable fraction was reduced (NaBD_4), permethylated and analyzed by gas-liquid chromatography-mass spectrometry. A single oligosaccharide component was seen with retention time and mass spectrum identical to β -D-GlcNAcp-(1-2)-[1-D]-glycerol. The non-dialyzable fraction gave only on sugar analysis D-Man and D-GlcNAc in the relative molar proportions 1:2, and methylation analysis showed that the D-Man residue was terminal and the D-GlcNAc residues were substituted in the 1- and 4-positions. The demonstration of β -D-GlcNAcp-(1-2)-[1-D]-glycerol in the dialyzable fraction shows that the oligosaccharide obtained by trifluoroacetylytic degradation of α_1 -microglobulin using TFA/TFAA, 1:1, must have the structure 1.

Trifluoroacetylysis of the non-dialyzable fraction using TFA/TFAA, 1:50, followed by de-O- and de-N-trifluoroacetylation by treatment with aqueous acetic acid and sodium borohydride yielded a product which after N-acetylation was fractionated by gel filtration (Fig. 1). Fractions were pooled as indicated in the figure. The component (1.8 mg) in pool A showed D-Man, D-GlcNAc, and D-GlcNAc-OL in the relative molar proportions 1:0.9:0.8 and had an optical rotation of -53° (c, 0.150, H_2O) demonstrating two β -linkages. The component (1.4 mg) in pool B showed D-Man and D-GlcNAc-OL in the relative molar proportions 1.0:0.9 and had an optical rotation of -59° (c, 0.120, H_2O) showing that the glycosidic linkage was β . The permethylated disaccharide alditol gave a mass spectrum similar to that of permethylated β -D-Galp-(1-4)-D-GlcNAc-OL as expected. From these results it is evident that the structure of the components in pool A and B are β -D-Manp-(1-4)- β -D-GlcNAcp-(1-4)-D-GlcNAc-OL and β -D-Manp-(1-4)-D-GlcNAc-OL, respectively.

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ISOLATION AND CHARACTERISTICS OF HUMAN URINARY SIALOGLYCOPROTEINS

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Summary

Three sialoglycoproteins (S_1 , S_2 , and S_3) have been isolated from normal human urine by ultrafiltration, zone electrophoresis, gel chromatography, and ion exchange chromatography. The average yields per litre of urine were 0.39 mg (S_1), 0.40 mg (S_2), and 1.9 mg (S_3). The isolated substances were homogeneous on ultracentrifugation both at neutral and acid pH with sedimentation coefficients of 3.6 S (S_1), 2.4 S (S_2), and 0.93 S (S_3). Equilibrium ultracentrifugation gave molecular weights of 77,900 (S_1), 37,000 (S_2), and 5,300 (S_3). All three components were rich in carbohydrate (64 to 69%) and contained 28 to 35% of sialic acid. Alkaline borohydride degradation of component S_3 yielded two sialylated oligosaccharides which were partially characterized.

The origin of the isolated substances is unknown. The molecular size of S_3 (Stokes' radius of 2.0 nm) is compatible with passage from the blood by glomerular filtration whereas the size of S_1 (Stokes' radius of 6.5 nm) would suggest a renal origin.

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Deceased in June 1978.

INTRODUCTION

Normal human urine is known to contain macromolecules characterized by a particularly high content of sialic acid (1). Only limited information is available concerning the chemical nature and origin of these substances. One of us (2) has previously reported the preparation by electrophoresis of a sialic acid-rich urinary glycoprotein fraction. The present paper describes the isolation from this material of three sialoglycoproteins (designated S_1 , S_2 , and S_3 , respectively). Some of the physical and chemical properties of the isolated substances are also given.

EXPERIMENTAL PROCEDURE

Materials

Urine from healthy male individuals was collected and pooled, and bacterial growth was prevented by addition of phenylmercuric nitrate to a final concentration of 1:50,000. The urine was filtered at 4°C, and immediately ultrafiltered.

Pevikon C-870 (Kema-Nord AB, Stockholm, Sweden), DEAE-cellulose (Serva Entwicklungslabor, Heidelberg, Germany), and Sephadex G-25, G-100, G-200 and Blue Dextran 2000 (AB Pharmacia, Uppsala, Sweden) were used according to the instructions furnished by the manufacturer. Rabbit anti-M and anti-N sera were produced in the Laboratory of Blood Groups, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland. Extract of Vicia graminea seeds was prepared

(3) at pH 6.5. Neuraminidase from Vibrio cholerae was obtained from Behringwerke AG, Marburg/Lahn, West Germany.

General Methods

Concentration by Ultrafiltration. The urinary macromolecules were concentrated by ultrafiltration with 23/32-inch dialysis tubing (Union Carbide Corp., Chicago, Ill., USA) (4). The concentrates were processed immediately or stored at -20°C .

Electrophoresis. Preparative zone electrophoresis in blocks of Pevikon C-870 were carried out as previously described (5-7). All experiments were done in 0.1 M sodium acetate buffer, pH 4.5.

Determinations of Stokes' Radii, Diffusion Coefficients, Sedimentation Coefficients, Molecular Weights, and Frictional Ratios. Stokes' molecular radii were estimated by gel chromatography (8) on a Sephadex G-200 column (100 x 1 cm), equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl. Diffusion coefficients ($D_{20,w}$) were calculated from Stokes' radii with the Stokes-Einstein formula (9). Measurements of sedimentation coefficients and molecular weights were performed at 20°C in a Spinco model E analytical ultracentrifuge. Sedimentation analyses were carried out in a synthetic boundary cell using Schlieren optics. Experiments were conducted at 59,780 rpm in 0.1 M Tris-HCl buffer pH 8.0, in 0.5 M NaCl, and in 1.0 M acetic acid. Calculations were made according to the method of Schachman (10).

Molecular weights were estimated by sedimentation equilibrium in a double sector cell using interference optics. These experiments were carried out in 0.1 M Tris-HCl buffer, pH 8.0.

Glycoproteins S_1 (0.1%) and S_2 (0.2%) were analyzed by the meniscus depletion technique of Yphantis (11) at 17,250 rpm and 15,220 rpm, respectively. The molecular weight of fraction S_3 (0.3%) was determined at 20,400 rpm using a low speed method (12). Partial specific volumes for the calculations were derived from the carbohydrate and amino acid compositions (13,14).

Svedberg's equation was used to obtain the molecular weight from the diffusion coefficient and the sedimentation coefficient. The frictional ratio (f/f_0) was calculated from the sedimentation coefficient and the molecular weight determined by sedimentation equilibrium ultracentrifugation. The formulae used have been reported by Svedberg and Pedersen (15).

Analytical Methods

Quantitative amino acid analyses were performed by the method of Spackman, Stein and Moore (16) using a Biochrom amino acid analyzer (Biocal, Munich, West-Germany) equipped with a micro cuvette. The samples (1-1.5 mg) were hydrolyzed in 6 M HCl at 110°C for 24 or 72 h (17).

Nitrogen was estimated by the method of Kirsten and Hozumi (18) at the Chemical Institute of the Agricultural University of Sweden, Ultuna, Uppsala. Moisture in lyophilized samples was determined by drying to constant weight at 100°C and 0.05 mm Hg.

Colorimetric methods were used for determination of hexose (19), 6-deoxyhexose (20), sialic acid (21), and protein (22).

Sugar analyses were performed by gas-liquid chromatography (23) and mass spectrometry (24). Optical rotations were recorded using a Perkin-Elmer 241 polarimeter.

Methods for methylation analyses and permethylation of

reduced oligosaccharides have been described elsewhere (25). A Perkin-Elmer model 3920 gas chromatograph was used under the following conditions: (a) glass column, 2 m x 0.5 cm, packed with 3% ECNSS-M on Gas-Chrom Q (100-200 mesh) at a column temperature of 190°C for sugar alditol acetates, (b) glass capillary column (25 m x 0.25 mm) wall-coated with SE-30 at 185°C for partially methylated alditol acetates and at 180-330°C for permethylated oligosaccharides. Gas-liquid chromatography-mass spectrometry was performed on a Varian MAT 311A instrument fitted with the same capillary column as above connected directly to the ion source of the instrument. The mass spectra were recorded at an ionization potential of 70 eV, an ionization current of 3 mA, and an ion source temperature of 120°C. All data were processed by an on-line computer system (Spectro-system 100 Varian MAT).

Alkaline Borohydride Degradation. O-Glycosidically linked oligosaccharides were released from component S₃ by treatment with alkaline borohydride. The procedure of Spiro and Bhoyroo (26) was followed in detail.

Partial Hydrolysis. Desialylation of intact component S₃ and of oligosaccharides released from component S₃ by alkaline borohydride treatment was accomplished by heating 1% solutions for 1 h at 80°C in 0.025 M H₂SO₄ or by treatment with neuraminidase in 0.1 M sodium acetate buffer, pH 5.0, using sialic acid:neuraminidase ratios of 1:2 (w/w) at 37°C for 24 h.

Serological Assays. Two-fold diluted 25 µl aliquots of anti-M and anti-N sera and V. graminea extract were treated with 25 µl of saline (control) or 1% solutions of components S₁, S₂, and S₃. After 30 min at room temperature 25 µl of 2%

suspensions of red cells were added and agglutination was measured after 20 min (rabbit antisera) or after 1 h (V. graminea extract). When a complete inhibition of agglutination was obtained, the twofold diluted samples of inhibitor mixed with undiluted agglutinin solution were tested in the same way.

RESULTS

Isolation of Urinary Sialoglycoproteins

Sialic acid-rich components were isolated separately from two portions of non-ultrafilterable urinary macromolecules. These portions were obtained from 39 litres and 31 litres of normal urine. The content of non-ultrafilterable protein and sialic acid per litre of urine was approximately 90 mg and 16 mg, respectively.

Aliquots (8-12 ml) of the concentrates were fractionated by zone electrophoresis. Fig. 1 shows the distribution of sialic acid and protein after electrophoresis. Most of the material containing sialic acid migrated rapidly and formed a broad fraction at the anodal end of the block. The protein curve showed a distinct peak near the origin. The eluates containing the maximum and fast-moving part of the sialic acid peak were combined, dialyzed against distilled water and lyophilized. The yield of sialic acid-rich substance at this stage was approximately 6 mg per litre of urine. The glycoprotein fraction obtained by electrophoresis was chromatographed on a Sephadex G-200 column equilibrated with 0.5 M NaCl. Fig. 2 shows the elution pattern of material prepared from 31 litres of urine. Sialic acid and protein analyses revealed the presence of three main fractions, designated S_1 , S_2 , and S_3 . All

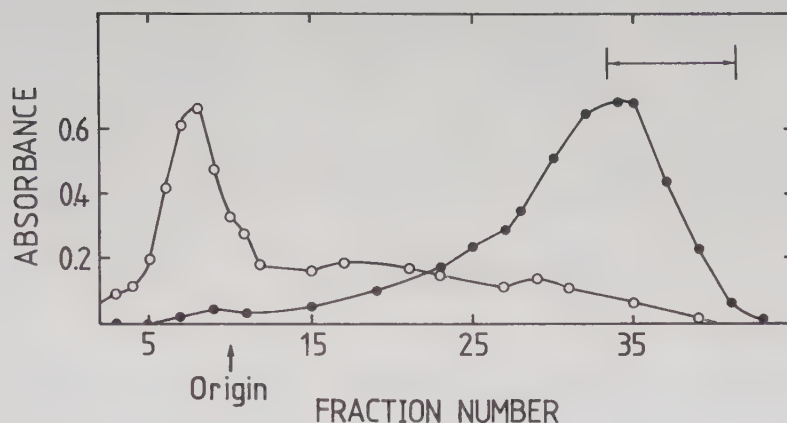


Fig. 1. Zone electrophoresis (0.1 M acetate buffer, pH 4.5, potential gradient 3.5 V/cm, 41 h) of concentrated macromolecules from 7 litres of normal urine. The material contained 110 mg of sialic acid and 630 mg of protein. The anode is to the right. Sialic acid-rich fractions were combined as marked on the figure. ●—● Sialic acid. ○—○ Protein.

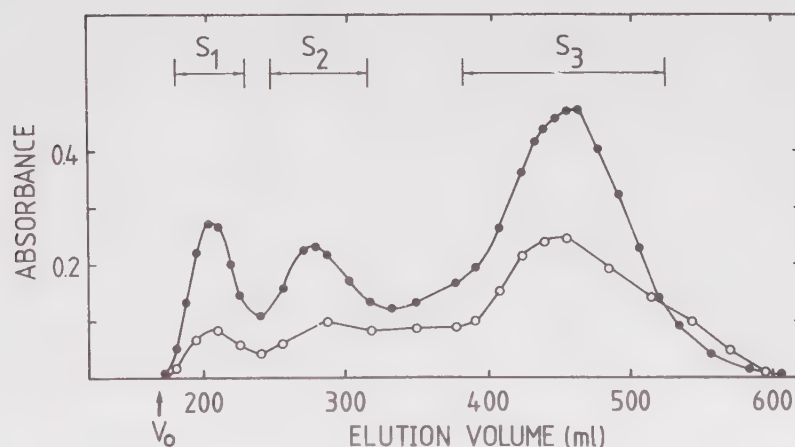


Fig. 2. Chromatography on Sephadex G-200 of sialic acid-rich material (194 mg) obtained by electrophoresis. The column (2.4 x 133 cm) was equilibrated with 0.5 M NaCl. Fractions of 7.5 ml were collected at 30 min intervals. V_0 indicates the void volume of the column. Eluates corresponding to fractions S_1 , S_2 , and S_3 were combined as shown in the figure. ●—● Sialic acid. ○—○ Protein.

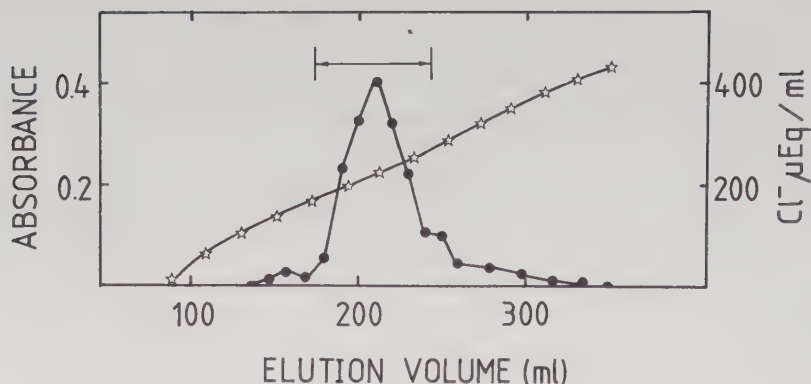


Fig. 3. Chromatography on DEAE-cellulose of fraction S_2 (18.4 mg) obtained by gel chromatography (Fig. 2). The column ($2^2 \times 6$ cm) was equilibrated with 0.1 M sodium acetate buffer, pH 4.5. Elution was carried out with a linear gradient. The mixing vessel contained 260 ml of 0.1 M sodium acetate buffer, pH 4.5, and the reservoir vessel 250 ml of the same buffer containing 0.6 M sodium chloride. Five ml portions of effluent were collected at 20 min intervals. ●—● Sialic acid. ☆—☆ Gradient of chloride ions. Fractions were pooled as indicated.

three fractions were retarded on the column. They were lyophilized after dialysis against distilled water. Fractions S_1 , S_2 , and S_3 were finally submitted to chromatography on DEAE-cellulose. Columns of the ion exchanger were equilibrated with 0.1 M sodium acetate buffer, pH 4.5. The various fractions were dialyzed against the same buffer prior to application, and elution was performed with a linear salt gradient as described in the legend of Fig. 3. The figure illustrates chromatography of fraction S_2 . Fractions S_1 and S_3 gave similar chromatograms. One reasonably symmetrical peak was observed in each case which contained most of the material applied to the column. After dialysis against distilled water, the three fractions were lyophilized and weighed. The yields of purified materials from the 31 l and 39 l samples of urine were as follows: fraction S_1 ,

Table 1. Physical properties of sialoglycoproteins S_1 , S_2 , and S_3 .

Parameter	S_1	S_2	S_3
Sedimentation coefficient, $s_{20,w}^o$ (sec $\times 10^{13}$)	3.6	2.4	0.93
Partial specific volume (ml/g) ^a	0.635	0.640	0.644
Stokes' radius (nm)	6.5	4.6	2.0
Diffusion coefficient, $D_{20,w}$ (cm ² /sec $\times 10^7$) ^b	3.25	4.6	10.6
Molecular weight			
by sedimentation equilibrium	77,900	37,000	5,300
by sedimentation-diffusion	74,900	35,500	5,900
Frictional ratio (f/f_o)	2.4	2.2	1.8
Absorption coefficient at 278 nm $10^3 M^{-1} cm^{-1}$	12.6	8.6	1.13

^a Calculated from carbohydrate and amino acid compositions.

^b Apparent diffusion coefficient calculated from Stokes' radius.

0.36 and 0.42 mg per litre of urine; fraction S_2 , 0.38 and 0.42 mg per litre; and fraction S_3 , 1.7 and 2.1 mg per litre.

Properties of Fractions S_1 , S_2 , and S_3

Table 1 shows some physical properties of fractions S_1 , S_2 , and S_3 . Sedimentation velocity experiments were performed at concentrations ranging from 0.1% to 1.3%. All three fractions behaved as single, homogeneous components in the ultracentrifuge. Most experiments were conducted in 0.5 M NaCl. Determinations carried out in 0.1 M Tris-HCl buffer, pH 8.0, and in 1 M acetic acid gave similar results. The sedimentation coefficient of S_3 seemed to be independent of concentration, whereas the sedimentation coefficients of S_2 , and particularly S_1 , decreased

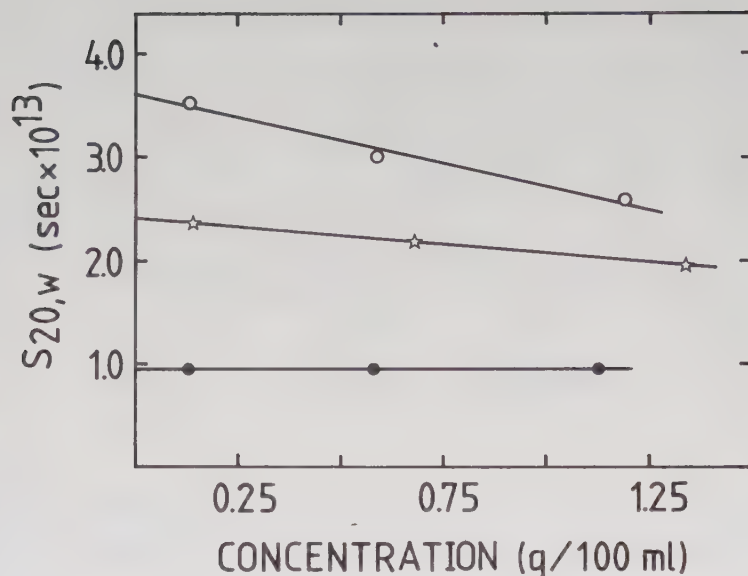


Fig. 4. Sedimentation coefficients of fractions S_1 (O—O), S_2 (★—★), and S_3 (●—●) as a function of concentration. Measurements were carried out in 0.5 M sodium chloride.

with increasing concentration (Fig. 4).

The molecular weight values obtained by equilibrium ultracentrifugations were similar to those calculated from sedimentation and diffusion coefficients. The frictional ratios are, no doubt, to a large extent due to the hydrophilic properties of the substances. At least the ratios of S_1 and S_2 , which were particularly high, may also indicate asymmetrical shapes. The ultraviolet absorption curves of the three components were very similar with plateaux in the region from 255 nm to 280 nm. The low absorption coefficients at 278 nm indicate low contents of tryptophan and tyrosine.

The nitrogen, polypeptide and the main classes of carbohydrate content of S_1 , S_2 , and S_3 is given in Table 2. It is apparent that the three substances have a similar gross composition. They are very rich in carbohydrate and contained as much

Table 2. Content of nitrogen, polypeptide, and carbohydrate in sialoglycoproteins S_1 , S_2 , and S_3 .

Constituent	S_1	S_2	S_3
	(g per 100 g) ^a		
Nitrogen	6.9	7.5	7.8
Polypeptide moiety ^b	23	27	35
Carbohydrate moiety	66	69	64
Total hexose	23.6	11.6	17.0
Hexosamine ^c	9.3	22.1	18.4
Sialic acid	32.7	35.2	28.4

^a All figures, except those for the polypeptide moieties of S_1 and S_2 , are average values from analyses of two preparations.

^b Calculated from quantitative amino acid analyses.

^c As N-acetyl derivative.

as approximately 30% of sialic acid. On the basis of the properties given in Tables 1 and 2, S_1 , S_2 , and S_3 have been labelled sialoglycoproteins.

Table 3 presents the detailed amino acid and carbohydrate contents of S_1 , S_2 , and S_3 on a molar basis. The amino acid compositions were different although some similarities were observed, particularly between S_1 and S_2 . Threonine was found in high amounts in all three substances. The amino acid analyses disclosed an uneven number of many residues, indicating heterogeneity, especially in S_3 .

Determination of M, N, and N_v_g Blood Group Activity of Urinary Sialoglycopeptides

Results from serological assays are given in Table 4. None

Table 3. Amino acid and carbohydrate composition of sialoglycoproteins S₁, S₂, and S₃. Except where noted, all figures are average values from one 24 h and one 72 h hydrolysis.

Constituent	S ₁	S ₂	S ₃
	(residues per molecule) ^a		
Lysine	3.93	3.17	0.11
Histidine	2.97	1.79	0.28
Arginine	1.49	1.46	0.30
Aspartic acid	8.70	7.88	3.04
Threonine ^b	35.7	17.7	2.92
Serine ^b	28.4	12.5	1.39
Glutamic acid	17.4	12.3	2.26
Proline	21.8	13.1	1.60
Glycine	11.2	5.09	1.04
Alanine	16.1	6.75	0.96
Half-cystine	-	trace	trace
Valine ^c	9.08	4.51	1.49
Methionine	3.53	0.74	0.08
Isoleucine ^c	4.73	2.25	0.21
Leucine	9.46	4.94	0.83
Tyrosine	0.89	0.67	0.31
Phenylalanine	0.83	0.61	0.56
N-Acetylglucosamine	23.0	12.7	2.58
N-Acetylgalactosamine	12.6	27.5	2.22
N-Acetylneuraminic acid	86.0	44.0	5.09
Galactose	85.6	21.1	4.19
Mannose	21.2	2.76	0.88
Fucose	6.48	2.78	0.47
Total	411	206	32.8

^a Calculations were based on the molecular weights obtained by equilibrium ultracentrifugations (cf. Table 1).

^b Values obtained by extrapolation to 0 hour of hydrolysis.

^c 72 h hydrolysis value.

Table 4. Inhibition of rabbit anti-M and anti-N sera and Vicia graminea extract by sialoglycoproteins S_1 , S_2 , and S_3 .

Fraction	Activity (mg/ml)		
	M	N	N_{V_g}
S_1	>10 ^a	>10	>10
S_2	>10	>10	>10
S_3	>10	>10	5
S_3 (desialylated)			1.25

^a >10 - no inhibition at concentration 10 mg/ml.

of the tested glycoproteins inhibited anti-M and anti-N rabbit immune sera at a concentration of 10 mg/ml. Nor did fractions S_1 and S_2 inhibit the V. graminea extract at the same concentration. Fraction S_3 showed N_{V_g} inhibitory activity. It completely inhibited an equal volume of V. graminea extract (titer 1:8) at a concentration of 5 mg/ml and this inhibitory activity was increased to 1.25 mg/ml by desialylation of the glycoprotein with neuraminidase.

Characterization of O-Glycosidically linked Oligosaccharides Released from Component S_3

Component S_3 (90 mg) was treated with alkaline borohydride as previously described (26) and the degradation products were fractionated on a Sephadex G-25 column. Eluted fractions were analyzed for total hexose and sialic acid. Four hexose-containing peaks were obtained, three of which also contained sialic acid (Fig. 5). The material was pooled as indicated (A-D). Further purification of the material in pools B and C

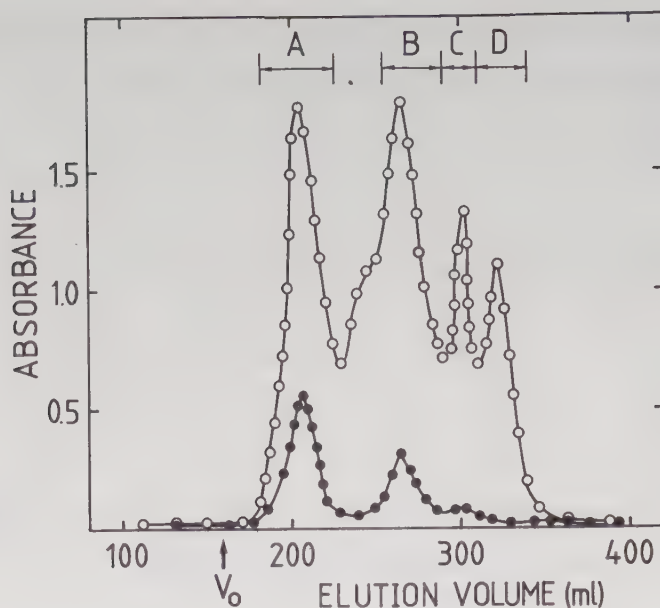


Fig. 5. Gel filtration on Sephadex G-25 of alkaline borohydride treated sialoglycoprotein fraction S_3 . The sample (31 mg) was placed on a column (2 x 130 cm), equilibrated with 0.1 M pyridine acetate buffer, pH 5.0. Fractions (5.8 ml) were collected at a flow rate of 10 ml/h. V_0 indicates the void volume of the column.

Fractions were pooled as shown (pools A-D). ●—● Sialic acid. O—O Total hexose.

was accomplished by ion exchange chromatography on DEAE cellulose. From each of pool B and C a symmetrical sialic acid-containing peak was eluted with a gradient of pyridine-acetate buffer (pH 5.4) 0.012 M and 0.060 M, respectively (27). Purified pools B and C were finally lyophilized and analyzed. Fraction B ($[\alpha]_D -11^\circ$) (c, 0.163, H_2O) yielded on sugar analysis D-galactose, N-acetyl-D-galactosamine, and N-acetylneuraminic acid in the relative molar proportions 1.0:0.7:2.1. Pool C ($[\alpha]_D -28^\circ$) (c, 0.065, H_2O) yielded the same sugars but in the relative molar proportions 1.0:1.0:0.9. The sugars in fractions B and C accounted for more than 95% of the material indicating that both are oligosaccharides.

Table 5. Methyl ethers obtained in methylation analyses of oligosaccharides in purified fractions B and C.

Sugars	T-value ^a	Relative molar proportions B	proportions C
2,3,4,6- <u>O</u> -Me- <u>D</u> -Gal	1.07	0.58	0.73
2,4,6- <u>O</u> -Me- <u>D</u> -Gal	1.55	0.42	0.27
1,4,5,6- <u>O</u> -Me- <u>D</u> -GalN(Me)Ac-OL ^b	2.10	-	+
1,4,5- <u>O</u> -Me- <u>D</u> -GalN(Me)Ac-OL	3.70	+	+

^a As their corresponding alditol acetates relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol.

^b 2-(N-methyl)-acetamido-2-deoxy-1,4,5,6-tetra-O-methyl-D-galactitol.

Compounds B and C were subjected to methylation analysis before enzymic removal of N-acetylneuraminic acid. The results are summarized in Table 5. Desialylated components B and C gave one single peak which on gas chromatographic-mass spectrometric analysis of the permethylated derivatives was identical with an authentic sample of reduced and permethylated β -D-Galp-(1-3)-D-GalNAc-OL-1 (28).

DISCUSSION

Sialoglycoproteins S_1 and S_2 have not been previously described. Sialoglycoprotein fraction S_3 has some resemblance to urinary glycopeptides isolated by Carrion *et al.* (29) and Goussault and Bourrillon (30). There are, however, definite differences between the two materials, such as in the fucose:sialic acid ratios.

The question whether S_1 , S_2 , and S_3 are related to each other might be raised on the basis of similarities in their gross chemical compositions. S_3 apparently is not a subunit of S_1 and (or) S_2 since it contains relatively more polypeptide of a different composition. A monomer-dimer relationship between S_2 and S_1 , which might be assumed from their molecular weights, is excluded by some distinct chemical differences, e.g. in content of methionine and in ratios of aspartic acid:threonine and glucosamine:galactosamine. S_1 and S_2 do not contain non-covalently linked subunits which can be dissociated in 1 M acetic acid.

It is not known whether the three isolated substances are excreted from the blood or if they derive from the kidney. It has been found that dextran fractions with molecular weights below 10,000-15,000 cross the glomerular membrane at the same rate as water, and that dextran of molecular weight 50,000-60,000 is practically excluded from the glomerular filtrate (31,32). The Stokes' molecular radii of these two molecular weight classes of dextran are about 2.5 nm and 5.0-5.5 nm, respectively (33). Similar molecular size limits are also valid for polyvinyl pyrrolidone (34). Accordingly, the size of S_3 (Stokes' radius of 2.0 nm) is easily compatible with glomerular filtration, whereas the size of S_1 (6.5 nm) would suggest a renal origin. Since S_1 , S_2 , and S_3 are charged substances their passage through membranes might be more restricted than the passage of the uncharged dextran and polyvinylpyrrolidone molecules (35).

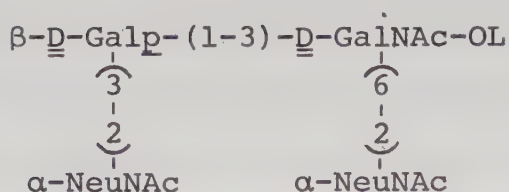
Some properties of sialic acid-rich glycoproteins isolated from human plasma have been published (36). The characteristics of these glycoproteins resemble those of urinary sialoglyco-

protein S_1 . Immunochemical methods should be suited for exploring the possible relationships between sialoglycoproteins in plasma and urine, although our attempts to produce antisera against S_1 and S_2 in rabbits have been unsuccessful. The chemical compositions of S_1 , S_2 , and S_3 are somewhat similar to those of human M and N blood group antigens isolated from erythrocytes (37,38). Accordingly, the possibility was considered that the urinary substances represent soluble M and N blood group antigens which might be derived from the catabolism of cell membranes. Serological tests did not reveal any M or N activity but the anti-N activity of the lectin from Vicia graminea seeds (N_{Vg} activity) could be inhibited by S_3 to an extent comparable to the N_{Vg} activity of tryptic glycopeptides and their cyanogen bromide fragments derived from M and N erythrocyte glycoproteins (39). The N_{Vg} activity, which is confined to alkali-labile oligosaccharide chains of M and N glycoproteins has previously been shown to be enhanced after release of sialic acid (39,40).

The chemical nature of the oligosaccharide chains in S_3 was studied after they had been released by alkaline borohydride treatment. Sugar analysis of the component in fraction B showed NeuNAc, $\underline{\underline{D}}$ -Gal, and $\underline{\underline{D}}$ -GalNAc-OL in the relative molar proportions 2:1:1 suggesting a tetrasaccharide structure. Methylation analysis demonstrated the structural elements $\underline{\underline{D}}$ -Galp-(1-, -3)- $\underline{\underline{D}}$ -Galp-(1-, and -3)- $\underline{\underline{D}}$ -GalNAc-OL in about equal proportions.

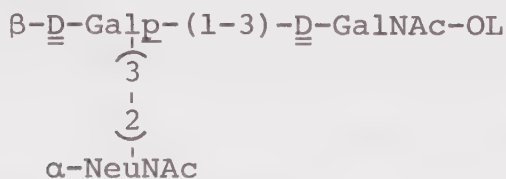
Desialylation of the fraction yielded a single component which was identified as β - $\underline{\underline{D}}$ -Galp-(1-3)- $\underline{\underline{D}}$ -GalNAc-OL by gas-liquid chromatography-mass spectrometry of its permethylated

derivative (28). The data suggest the structure

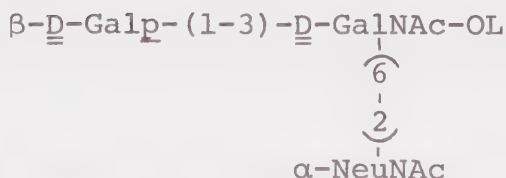


for the component in pool B. The presence of $\underline{\underline{\text{D}}}\text{-Galp-(1-}$ in the methylation analysis indicates that the component had lost 60% of the NeuNAc-(2-3)- residues on storage.

Pool C showed on sugar analysis NeuNAc, $\underline{\underline{\text{D}}}\text{-Gal}$, and $\underline{\underline{\text{D}}}\text{-GalNAc-OL}$ in the relative molar proportions 1:1:1 suggesting a trisaccharide structure. Methylation analysis demonstrated the structural elements $\underline{\underline{\text{D}}}\text{-Galp-(1-}$ and $\text{-3)-}\underline{\underline{\text{D}}}\text{-Galp-(1-}$ in about equal proportions and also the structural units $\text{-3)-}\underline{\underline{\text{D}}}\text{-GalNAc-OL}$ and $\text{-3)-}\underline{\underline{\text{D}}}\text{-GalNAc-OL}$. Desialylation of fraction C yielded a single component $\begin{array}{c} \text{6} \\ | \\ \text{1} \end{array}$ which was identified as $\beta\text{-}\underline{\underline{\text{D}}}\text{-Galp-(1-3)-}\underline{\underline{\text{D}}}\text{-GalNAc-OL}$ by gas-liquid chromatography-mass spectrometry of its permethylated derivative (28). The data presented suggest that pool C consists of a mixture of the trisaccharides:



and



The anomeric configurations were deduced from optical rotation data and information obtained after treatment with α -neuraminidase. The results are in accordance with the struc-

ture shown for alkali-labile chains of M and N glycoproteins (37). This structure is also present in several other glycoproteins (28,29,41) and was found in a glycoside of serine isolated from human urine (42). The origin of the urinary S_1 , S_2 , and S_3 is unknown but available information suggests that they originate from catabolism of cellular and mainly surface membrane glycoproteins.

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